clarify the molecular events involved in hepatic differentiation.

In this study, we show that the gene expression pattern of ES cell–derived hepatocytes is similar to that in adult mouse liver. Furthermore, by using small interfering RNA (siRNA) technology, we demonstrate that HNF3β/FoxA2 plays a critical role in early hepatic differentiation from ES cells. These results suggest that the HIFC differentiation system allows hepatic differentiation of ES cells via endoderm differentiation, thus recapitulating the *in vivo* liver developmental program.

Materials and Methods

The HIFC Differentiation System and Culture of ES Cells. pALB-EGFP/ES cells, a J1 cell clone of 129X1/SvJ male origin, were cultured as previously described.[8,9] The HIFC differentiation system was performed as follows: undifferentiated ES cells were treated for 3 days in an ES cell culture medium containing leukemia inhibitory factor (100 U/mL) and 10^{-8} mol/L *all-trans*-retinoic acid; for 5 days with a leukemia inhibitory factor (−) culture medium in the presence of a growth factor combination (fibroblast growth factor 1, 100 ng/mL; fibroblast growth factor 4, 20 ng/mL; hepatocyte growth factor, 50 ng/mL [VERITAS, Tokyo, Japan]) in gelatin-coated dishes (Asahi Techno Glass, Chiba, Japan); and for 2 days with a leukemia inhibitory factor (−) culture medium in the presence of oncostatin M (10 ng/mL) (VERITAS) in type I collagen–coated dishes (Asahi Techno Glass). Differentiated hepatocytes were identified as green fluorescent protein (GFP)-positive. GFP gene expression was monitored via fluorescence microscopy (Nikon, Tokyo, Japan).

Isolation of Total RNA. Total RNA was extracted from undifferentiated ES cells, HIFC-treated cells, and HIFC-untreated cells using ISOGEN solution (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol and treated with deoxyribonuclease (DNase I, amplification grade; TaKaRa, Kyoto, Japan).

Microarray Analysis. AceGene mouse oligo chip subsets A and B (DNA Chip Research and Hitachi Software, Yokohama, Japan) DNA microarrays were used according to the manufacturer's instructions (http://www.dna-chip.co.jp/thesis/AceGeneProtocol.pdf). MIAME (minimum information about a microarray experiment) guidelines were applied for microarray data analysis.[10] RNA was extracted from day 0 (undifferentiated ES cells), day 6 (+) (HIFC-treated sample), day 6 (−) (HIFC-untreated sample), day 10 (+) (HIFC-treated sample), and day 10 (−) (HIFC-untreated sample) cells and 129X1/SvJ mouse liver (8 weeks old). As a total RNA reference, equal amounts of day 0, day 6 (+), day 6 (−), day 10 (+), and day 10 (−) RNA were mixed together. To ensure data reliability, weak signal spots were removed according to the following criteria. For each sample, the experiment was repeated once, wherein the dye was reversed between the experimental and the reference sample to account for dye-incorporation bias. This resulted in a data matrix of 9,172 genes that contained no missing data.

Genes [day 10 (+) sample data] that showed an increase or decrease in their expression levels of more than twofold compared with day 10 (−) sample data were designated as upregulated and downregulated genes, respectively. A hierarchical cluster was produced from upregulated and downregulated gene data using a Euclidean distance calculation based on the Unweighted Pair Group Method with Arithmetic Mean by GenMaths software (Applied Maths). Gene ontology (GO) categories were assigned to genes based on the AceGene microarray database (DNA Chip Research and Hitachi Software, Yokohama, Japan). The significance of GO term appearance in the upregulated and downregulated genes (compared with all genes: 9,172 genes) was calculated as the *P* value using the software GO Term Finder adapted to the AceGene microarray (http://db.yeastgenome.org/cgi-bin/SGD/GO/goTermFinder). Cutoff points were set at 0.005.[11] By comparing the day 10 (+) and day 10 (−) and focusing on genes with twofold or more alteration in expression level, we reasoned that these gene profiles represented the main cell population (GFP-positive cells) and not the minority cell populations (GFP-negative cells).

Reverse-Transcription Polymerase Chain Reaction. Total RNA (1 μg) was reverse-transcribed using the SuperScript II First-Strand Synthesis System (Invitrogen, Tokyo, Japan) according to the manufacturer's protocol. Polymerase chain reaction (PCR) reactions were performed using the Ampli Taq Gold kit (Roche Diagnostics, Tokyo, Japan). PCR primers and conditions are listed in Table 1.

Transfection With HNF3β/FoxA2 siRNA. Synthetic 21-nt RNAs were purchased from QIAGEN (Tokyo, Japan) in deprotected, desalted, and annealed form. The sequences of our prepared mouse HNF3β/FoxA2 siRNAs are listed in Table 2. Control luciferase GL2 siRNA was purchased from Dharmacon (Boulder, CO). Twenty-four hours before transfection with the ES cell culture medium, 1×10^4 cells/mL of regularly passaged ESJ1 pALB-EGFP cells were plated. Lipofectamine 2000-mediated transient transfection of siRNA was performed on days 1 and 3 as described by the manufacturer

(Invitrogen). GFP-positive cells were monitored on day 7 via fluorescence microscopy (Nikon).

ES cells were transfected with an Alexa-labeled siRNA (QIAGEN, Tokyo, Japan) in the same manner. Five hours after transfection, the cells were washed with phosphate-buffered saline and observed via fluorescence microscopy (Nikon) at ×100 magnification. Alexa-positive cells and total cells were counted in five randomly selected fields. The transfection efficiency was calculated as a percentage of Alexa-positive cells per well.

Table 1. Primers and Conditions for RT-PCR and Real-Time PCR

GeneBank Accession No.	Primers	Annealing Temperature (°C)	Cycles
RT-PCR			
NM009327	HNF1α S: 5′-GCCTAATGGCCTTGGAGAAA-3′ A: 5′-TCTTAGTTGGCAGCTCATCG-3′	64	36
NM009330	HNF1β S: 5′-ACCAAGCCGGTTTTCCATAC-3′ A: 5′-ATCTTCTTGTTGGTGGGCTC-3′	64	32
NM008259	HNF3α S: 5′-GTGAAGATGGAAGGGCATGA-3′ A: 5′-TCGTGATGAGCGAGATGTAG-3′	64	36
NM010446	HNF3β S: 5′-CGCCAACATGAACTCGATGA-3′ A: 5′-CTTAGGCCACCTCGCTTGTG-3′	58	30
NM008260	HNF3γ S: 5′-TGAAGATGGAGGCTCATGAC-3′ A: 5′-CAAACATGTTCCCAGAGCTG-3′	64	32
NM008261	HNF4α S: 5′-ATTCATCCAACAGCCTGAGC-3′ A: 5′-CGTCTGTGATGTTGGCAATC-3′	64	36
NM008262	HNF6 S: 5′-CAAATGGCTTTGAAGCCCAC-3′ A: 5′-TGCTTTGGTACAAGTGCTCG-3′	60	32
NM007678	C/EBPα S: 5′-CAACGCCCGCCTTTGGCTTT-3′ A: 5′-GCGTCTCCACGTTGCGTTGT-3′	56	30
NM009883	C/EBPβ S: 5′-CGGATCAAACGTGGCTGAG-3′ A: 5′-CGCAGGAACATCTTTAAGGTGA-3′	56	32
NM008092	GATA-4 S: 5′-CAAGATGAACGGCATCAACC-3′ A: 5′-CACAGTACTGAATGTCTGGG-3′	60	32
NM010258	GATA-6 S: 5′-CATCACCATCACCCGACC-3′ A: 5′-TTCATATAGAGCCCGCAAGC-3′	58	32
AF440692	Trf S: 5′-ACTGCCATTCGGAATCA-3′ A: 5′-CGGCATAATACCCTTTAGGA-3′	56	30
NM013474	Apoa2 S: 5′-CACTGCTGGTAACCATCTG-3′ A: 5′-TCGAGGTTCATTAAACTGCT-3′	56	30
X62595	Cyp2e1 S: 5′-GTCAGAGGCGCATCGT-3′ A: 5′-CAGCCAATCAGAAAGGTAGG-3′	54	40
NM007376	Pzp S: 5′-TACTTGGCTCACTGCGTTTG-3′ A: 5′-CGCTTCTCTTCGCCTTGTT-3′	54	36
X04115	Mup1 S: 5′-GCTCCGAATTATCTATGGTT-3′ A: 5′-TGGCATTGGATAGGTCA-3′	54	30
NM009976	Cst3 S: 5′-GTTCCTGCTGGCCGTCCT-3′ A: 5′-GGCTGTGGTACGCATCGTTG-3′	60	30
AK012563	Anxa2 S: 5′-CATTGCCTTCGCCTATCAGA-3′ A: 5′-GGCATCCTGGTAATCAGC-3′	57	30
NM007397	Acvr2b S: 5′-CCGGCATGAAGCACGAAA-3′ A: 5′-CCCTGGCTCAAACCGAACAG-3′	60	30
NM007393	βactin S: 5′-AGAGCAAGAGAGGTATCCTG-3′ A: 5′-GCAGAAGCCTAGTTGGATCA-3′	56	25
Real-time PCR			
NM010446	HNF3β S: 5′-CGAGAACGGCTGCTACCTG-3′ A: 5′-GGTCTTCTTGCCTCCGCTAC-3′	58	50
AJ011413	ALB S: 5′-AAGCTGAGACCTTCACCTTC-3′ A: 5′-CAGCAGCCTTGCAACATGTA-3′	55	50
BC024702	TTR S: 5′-AAAGACCTCTGAGGGATCCT-3′ A: 5′-CAGAGTCGTTGGCTGTGAAA-3′	55	50
5U00445	G6Pase S: 5′-CGCAGCAGGTGTATACTATG-3′ A: 5′-TTTCAGCCACAGCAATGCCT-3′	55	50
AK081405	GAPDH S: 5′-ATCACTGCCACCCAGAAGAC-3′ A: 5′-CACATTGGGGTAGGAACAC-3′	58	50

SYBR Green Real-Time PCR. Real-time RT-PCR primers are listed in Table 1. The complementary DNAs were used for PCR using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) in triplicate. Optimization of the real-time PCR reaction was performed according to the manufacturer's instructions (PE Applied Biosystems, Tokyo, Japan). All quantitations were normalized to an endogenous control GAPDH. Relative gene induction values were calculated according to the manufacturer's instructions.

Statistical Analysis. The results are given as the mean ± SD. Statistical analysis was conducted using ANOVA with the Bonferroni correction for multiple comparisons. A P value of .05 or less was considered significant.

Results

Differentiation and Microarray Analysis of ES Cell–Derived Hepatocytes. We previously established and reported the HIFC differentiation system,[7] whereby ES cells could be differentiated in 10 days into hepatocytes without forming embryoid bodies at a rate of 29.6% ± 1.8% (data not shown). Therefore, our novel culture system represents a reproducible model for studying the molecular mechanisms underlying hepatic differentiation *per* se.[7] To analyze genes related to hepatic differentiation, a profile of day 10 cells treated with HIFC [10 (+)] was compared with that of day 10 cells without HIFC [10 (−)]. Of the 9,172 genes analyzed, 232 genes showed a significant twofold or more alteration of the expression level, indicating that expression levels of these genes were altered by HIFC treatment.

Of the 232 genes, 30 of the most highly upregulated and downregulated genes are listed in Tables 3 and 4, respectively. Genes upregulated during the hepatic differentiation of ES cells comprised many metabolic enzymes, such as cytochrome P450 and alcohol dehydrogenase,[12]

Table 2. Sequences of HNF3β/FoxA2 siRNA

Name	Sequence
HNF3β/FoxA2-siRNA1	5′-CCUCCCUACUCGUACAUCUdTdT-3′ 3′-dTdTGGAGGGAUGAGCAUGUAGA-5′
HNF3β/FoxA2-siRNA2	5′-GCAACUGGCACUGAAGGAAdTdT-3′ 3′-dTdTCGUUGACCGUGACUUCCUU-5′
HNF3β/FoxA2-siRNA3	5′-UGGACCUCAAGGCCUACGAdTdT-3′ 3′-dTdTACCUGGAGUUCCGGAUGCU-5′
HNF3β/FoxA2-siRNA4	5′-GGCCUACGAACAGGUCAUGdTdT-3′ 3′-dTdTCCGGAUGCUUGUCCAGUAC-5′

Table 3. Upregulated Genes in ES Cell-Derived Hepatocytes

GeneBank Accession No.	Gene	Symbol	Fold	
			Day 10(+)	Liver
AF440692	Transferrin	Trf	35.377	13.563
X04115	Major urinary protein 1	Mup1	33.867	7.845
D83073	Mitogen-activated protein kinase 14	Mapk14	33.478	8.692
NM013474	Apolipoprotein A-II	Apoa2	33.023	10.900
AY073538	Olfactory receptor MOR145-4	MORI45-4	25.915	6.109
BC019965	Similar to major urinary protein 4		24.419	11.530
Y127132	Endogenous retroviral sequence 4 (with leucine t-RNA primer)	Erv4-pending	22.091	0.991
NM021528	Chondroitin 4-sulfotransferase 2	C4st2-pending	21.440	6.360
NM009247	Serine protease inhibitor 1-5	Spi1-5	20.045	7.363
NM013697	Transthyretin	Ttr	18.919	7.691
X13060	Albumin 1	Alb1	18.900	8.744
NM009244	Serine protease inhibitor 1-2	Spi1-2	18.129	8.390
X62595	Cytochrome P450, family 2, subfamily e, polypeptide 1	Cyp2e1	15.869	7.464
AK019146	Putative homeodomain transcription factor	Phtf	14.187	5.366
BC017687	Ubiquinol-cytochrome c reductase core protein 1	Uqcrc1	12.341	4.775
NM007443	Alpha 1 microglobulin/bikunin	Ambp	11.448	4.454
NM007376	Pregnancy zone protein	Pzp	11.136	31.266
NM007469	Apolipoprotein C-I	Apoc1	10.581	24.366
AF307745	Tyrosine kinase, nonreceptor, 1	Tnk1	9.428	3.308
AF403567	Aldolase 2, B isoform	Aldo2	8.663	4.202
J00369	Complement component 3	C3	8.499	13.565
NM010726	Phytanoyl-CoA hydroxylase	Phyh	7.227	2.825
AK021254	RIKEN cDNA C430046P22 gene	C430046P22Rik	7.024	22.691
NM010764	Mannosidase 2, alpha B1	Man2b1	6.985	2.779
NM007409	Alcohol dehydrogenase 1 (class I)	Adh1	6.440	10.540
BC013343	4-hydroxyphenylpyruvic acid dioxygenase	Hpd	6.264	14.715
NM134143	Cytochrome P450, family 2, subfamily c, polypeptide 40	Cyp2c40	6.143	9.015
AL136158	Dm538m10.1		6.002	1.818
AK018115	RIKEN cDNA 6330404E16 gene	6330404E16Rik	5.969	20.298
NM009381	Thyroid hormone responsive spot14 homolog	Thrsp	5.830	9.857

NOTE. Two hundred genes show the increase in their expression levels of more than twofold compared with day 10(−). Thirty of the most upregulated genes are listed.

and serum proteins, such as transthyretin, albumin, and major urinary protein 1 (Table 3).[13,14] On the other hand, downregulated genes in the hepatic differentiation of ES cells contained glycolysis-related proteins such as phosphoglycerate kinase 1 and lactate dehydrogenase 1, A chain (Table 4). Additionally, many of the 232 genes in the ES cell–derived hepatocytes are cell growth–, proliferation-, and physiology-associated genes. This includes 51 genes for cellular metabolism, 53 genes related to development, morphogenesis and differentiation, and 14 genes encoding cell–cell signaling and cell adhesion. Furthermore, several ES cell marker genes containing Oct3/4 and Nanog were downregulated as measured via microarray profiles and RT-PCR analysis (data not shown). Collectively, these results suggest that HIFC treatment induced differentiation from ES cells into cells with a gene expression profile typical of differentiated hepatocytes.

Clustering Analysis of ES Cell–Derived Hepatocytes. Unsupervised hierarchical cluster analysis was performed to sort the 232 altered genes (Fig. 1A). The case cluster analysis of microarray data revealed a striking mirroring of gene clusters between adult liver and day 10 (+) cells, indicating that ES cell–derived hepatocytes were similar to adult mouse liver in another aspect of the gene expression pattern. Interestingly, these analyses showed that significant gene expression alterations occurred during each stage of the process of hepatic differentiation [day 0, day 6 (+), and day 10 (+)], suggesting that ES cells differentiated into mature hepatocytes from nonhepatic precursor cells. The hierarchical clustering method delineated two distinctive major clusters according to the following expression patterns: group A (downregulated genes), consisting of 32 genes, and group B (upregulated genes), consisting of 200 genes (Fig. 1A).

To validate the results of the microarray analysis, a random selection of genes that were upregulated and downregulated in day 10 (+) cells was analyzed. As shown in Fig. 1B, the expression of upregulated genes Trf, Apoa2, and Cyp2e1 and downregulated genes Cst3, Anxa2, and Acvr2b was confirmed via RT-PCR. These results confirm the accuracy of the transcriptional regulation that was acquired from the microarray experiments.

GO Classification of ES Cell–Derived Hepatocytes. Using the database, the microarray analysis data were in-

Table 4. Downregulated Genes in ES Cell-Derived Hepatocytes

GeneBank Accession No.	Gene	Symbol	Fold	
			Day 10(+)	Liver
S74315	Antigen 1ec-a		0.275	0.517
U58494	Gag		0.296	0.582
AK021267	RIKEN cDNA C530002L11 gene	C530002L11Rik	0.333	0.550
NM007763	Cysteine rich intestinal protein	Crip	0.344	0.606
NM009976	Cystatin C	Cst3	0.356	0.587
AB071988	H2A histone family, member Y	H2afy	0.362	0.276
NM008492	Lactate dehydrogenase 2, B chain	Ldh2	0.362	0.133
AK012563	Annexin A2	Anxa2	0.382	0.646
AK003175	Tropomyosin 1, alpha	Tpm1	0.406	0.711
AY073382	Olfactory receptor MOR258-3	MOR258-3	0.413	0.573
NM009477	Uridine phosphorylase	Upp	0.413	0.130
NM015730	Cholinergic receptor, nicotinic, alpha polypeptide 4	Chrna4	0.428	0.794
NM024427	Tropomyosin 1, alpha	Tpm1	0.436	0.521
AK002214	WW domain-binding protein 5	Wbp5	0.445	0.669
NM007397	Activin receptor IIB	Acvr2b	0.447	0.313
NM030598	Down syndrome critical region gene 1-like 1	Dscr1l1	0.452	0.793
NM022985	Associated with Prkcl1	Awp1-pending	0.453	0.891
BC012446	Unknown protein		0.454	0.680
NM023409	Niemann Pick type C2	Npc2	0.455	0.372
NM009760	BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP3	Bnip3	0.458	0.331
NM008828	Phosphoglycerate kinase 1	Pgk1	0.442	0.156
NM008471	Keratin complex 1, acidic, gene 19	Krt1-19	0.465	0.382
BC024558	Hypothetical protein MGC37636	MGC37636	0.469	0.752
NM009426	Thyrotropin-releasing hormone	Trh	0.472	0.530
D86728	Heterogeneous nuclear ribonucleoprotein A1	Hnrpa1	0.483	0.639
NM010699	Lactate dehydrogenase 1, A chain	Ldh1	0.483	0.573
NM009811	Caspase 6	Casp6	0.489	0.641
AK005211	RIKEN cDNA 1500011H22 gene	1500011H22Rik	0.489	0.322
AK003007	RIKEN cDNA 0710007A14 gene	0710007A14Rik	0.494	0.644
X1695	Histone h2a		0.497	0.792

NOTE. Thirty-two genes show the decrease in their expression levels of more than twofold compared with day 10(−). Thirty of the most upregulated genes are listed.

tegrated to identify the GO biological processes for the upregulated and downregulated genes. This analysis indicated that four GO groups were highly significant for the upregulated and downregulated genes vis-à-vis the parent population (9,172 genes) (Table 5). The probabilities of observing such a number of genes in these categories by chance were extremely small, ranging from 3.7×10^{-4} to 2.5×10^{-3}. Interestingly, all of these GO groups are relevant to hepatocyte function, reinforcing the conclusion that hepatocyte-related genes were induced by HIFC treatment. For example, electron transport contains CYP 2e1 and CYP 2d10 genes. Thus, the result of GO analysis suggests that hierarchical clustering analysis of the hepatic differentiation of ES cells reflects a gene expression pattern similar to that of normal mouse liver.

Expression Pattern of Liver-Enriched Transcriptional Factors. To elucidate the gene expression profile of endodermal and hepatic transcription factors in the HIFC differentiation system, RT-PCR analysis was performed. Endoderm-associated transcription factors HNF1β/TCF2, HNF3α/FoxA1, HNF3β/FoxA2, and HNF4α were upregulated from day 1 during HIFC differentiation (Fig. 2A). These genes were not expressed in undifferentiated ES cells (day 0) and were not detected until day 3 in the HIFC-untreated cells (Fig. 2A). In contrast, the hepatocyte-enriched transcription factors HNF6/OC-1 and CCAAT/enhancer-binding proteins α and β were upregulated at the hepatocyte maturation stage (day 6 and thereafter) in HIFC-treated cells (Fig. 2B), whereas control cells did not show any upregulation of these transcription factors at any stage. These results indicated that endoderm-associated transcription factors were expressed at the early stage of the HIFC differentiation system and that, during the late stage of this system, hepatocyte-enriched transcription factors required for the maturation of hepatocytes were induced. These data suggest that HIFC differentiation directs ES cells to generate mature hepatocytes via endoderm differentiation.

HNF3β/FoxA2 Signaling Is Essential for Hepatic Differentiation of ES Cells. To further confirm that lineage-specific transcription factor networks were regulated in the HIFC differentiation system and that ES cells differentiated into hepatocytes via endoderm differentiation, we disrupted the expression of the endoderm-asso-

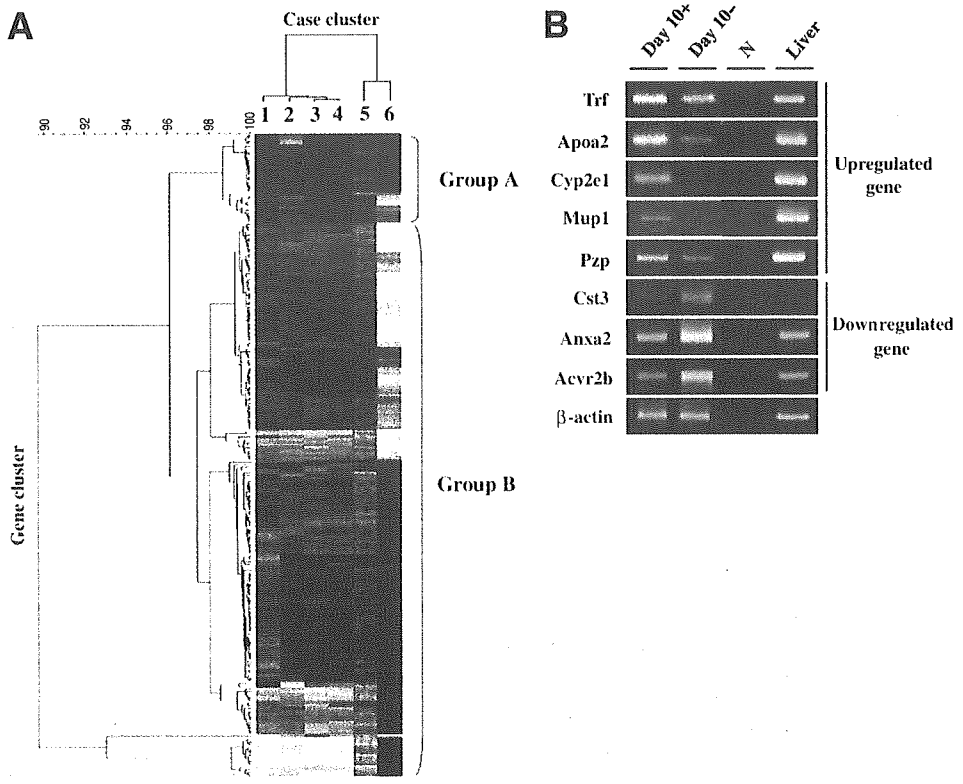

Fig. 1. Unsupervised hierarchical analysis of 232 gene expression profiles. (A) Data were subjected to a hierarchical cluster analysis using a Euclidean distance calculation based on the Unweighted Pair Group Method with Arithmetic Mean. The samples were aligned horizontally: lane 1, day 10 (−); lane 2, day 0; lane 3, day 6 (+); lane 4, day 6 (−); lane 5, day 10 (+); and lane 6, normal mouse liver complementary DNA. Samples were linked by the dendrogram shown above to highlight similarity in their gene expression patterns. The expression profile of each gene is depicted in the respective row. Genes are linked by the dendrogram shown on the left to highlight similarities in their expression patterns. The bracket on the right represents the identified clusters—namely, groups A and B. Red, black, and green areas represent high, middle, and low expression levels, respectively. (B) Verification of microarray analysis via RT-PCR. RT-PCR analysis was performed under linear amplification conditions. The β-actin gene was used as an internal control. Gene targets were randomly selected from the microarray analysis data. N = negative control.

ciated transcription factor HNF3β/FoxA2, which is expressed during the early stage of the HIFC differentiation system (Fig. 2A). First, the transfection efficiency of synthetic siRNA in ES cells was estimated to be approximately 70% using Alexa-labeled siRNA detectable via fluorescence microscopy (Fig. 3A). Second, we chose several siRNAs targeted against several sequences in the coding region of mouse HNF3β/FoxA2. HNF3β/FoxA2-siRNAs were transfected with liposome on days 1 and 3 in the HIFC-differentiation system. Among tested siRNA species, the HNF3β/FoxA2-siRNA1 treatment showed the most significant effect, with over 60% inhibition of HNF3β/FoxA2 expression levels, compared with the luciferase-siRNA control, by real-time PCR analysis (Fig. 3B). Therefore, HNF3β/FoxA2-siRNA1 was selected for further studies.

We investigated whether loss of HNF3β/FoxA2 expression affects the hepatic differentiation of ES cells by examining the effect of HNF3β/FoxA2-siRNA1 on the GFP-positive rate in the siRNA-transfected cells. GFP expression depends on albumin (ALB) expression under the control of the ALB promoter. As shown in Fig. 4A,

Table 5. Significance of Gene Ontology Appearance in Upregulated and Downregulated Genes

GO Term (Biological Process)	Cluster Frequency*	Sample Frequency of Use†	*P* Value
Blood coagulation	6 out of 183 genes (3.3%)	17 out of 6,699 genes (0.3%)	.00037
Response to pest/pathogen/parasite	13 out of 183 genes (7.1%)	103 out of 6,699 genes (1.5%)	.00038
Lipid transport	6 out of 183 genes (3.3%)	22 out of 6,699 genes (0.3%)	.0020
Electron transport	14 out of 183 genes (7.7%)	140 out of 6,699 genes (2.1%)	.0025

NOTE. The significance of GO term (biological process) appearance in upregulated and downregulated genes was calculated as a *P* value by the software.
*Of 232 genes, GO biological process is known for 183. Others are unknown.
†Of 9,172 genes, GO biological process is known for 6,699. Others are unknown.

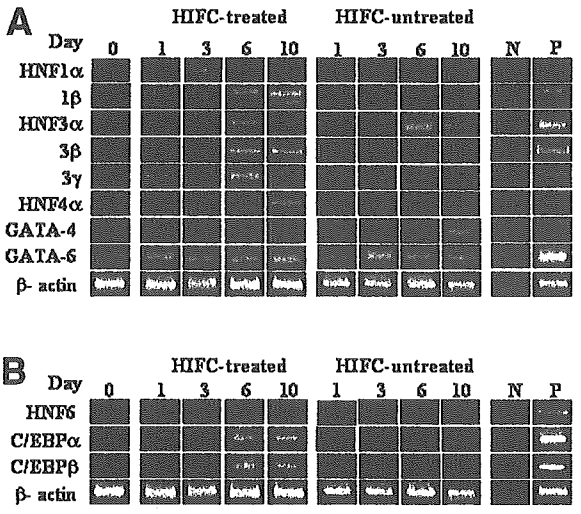

Fig. 2. Liver-enriched transcription factor gene expression analysis. Total RNA was isolated from undifferentiated ES cells (day 0), HIFC-treated cells (days 1, 3, 6, 10), and HIFC-untreated cells (days 1, 3, 6, 10). (A) RT-PCR analysis of endoderm-associated transcription factor genes. (B) RT-PCR analysis of liver-enriched transcription factor genes. As a quality control for messenger RNAs, PCR for β-actin was performed. N = negative control. P, mouse liver complementary DNA (8 weeks old) = positive control. HIFC, hepatic induction factor cocktail.

GFP-positive cells were observed in both HNF3β/FoxA2 siRNA1–transfected and luciferase siRNA–transfected cells. However, the HNF3β/FoxA2 siRNA1–transfected cells showed a greater than 60% decrease in GFP-positive rate compared with luciferase siRNA–transfected cells (Fig. 4B). This finding shows that differentiation of ALB-positive cells is suppressed in HNF3β/FoxA2 siRNA1–transfected cells. Additionally, real-time RT-PCR analysis showed that expression levels of transthyretin and glucose-6-phosphatase, which were typically expressed in mature hepatocytes, were also downregulated as well as albumin in the HNF3β/FoxA2 siRNA1–transfected cells (Fig. 4C). These results suggest that transcription factor networks are regulated precisely in the HIFC differentiation system and that ES cells differentiate into mature hepatocytes via endoderm differentiation.

Discussion

During mouse development, the first morphological sign of hepatogenesis is at 8.5 days postcoitum, when the visceral floor of the foregut endoderm thickens to form the liver diverticulum.[15] Tissue transplantation studies have shown that signals from the cardiac mesoderm induce the hepatic differentiation of the endoderm.[16,17] Cells of the hepatic endoderm then begin to migrate in a cord-like fashion into the surrounding mesenchyme of the septum transversum. There, the cords intermingle with the vitelline veins, which themselves begin to anastomose into a venous bed where the liver begins to form.[18] Just before birth and shortly thereafter, a large number of liver metabolic enzymes are induced. After birth, the liver acquires additional metabolism functions and becomes fully mature.[19] To understand the molecular mechanism for liver development *in vitro*, previous studies have attempted to show that ES cells differentiate into hepatocytes by forming embryoid bodies *in vitro*.[20-23] None of the reports, however, elucidated the molecular mechanisms underlying the differentiation of ES cells into hepatocytes, because the formation of embryoid bodies is not easily controlled. In this study, using the HIFC differentiation system, our data clearly demonstrated the applicability of microarray analysis and the controlled and reproducible hepatic differentiation of ES cells. Moreover, analysis of GO groups indicated that significant categories of GO appearance in 232 genes contained categories relevant to hepatic function. This integrative perspective on the gene expression profile validates our HIFC differentiation method for hepatic induction from ES cells. Therefore, the microarray analysis readout provides a potentially valuable resource for further detailed dissection of key molecules and demonstrates that these

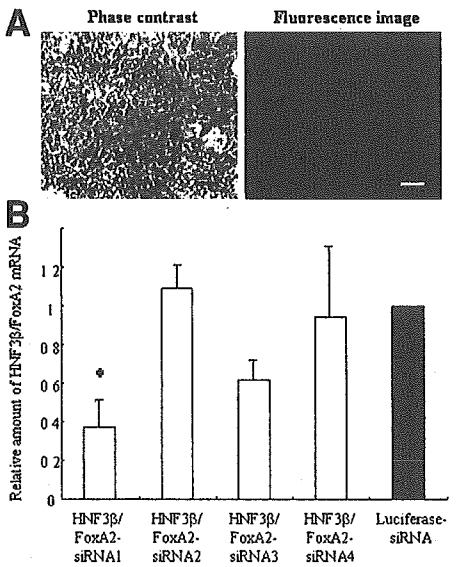

Fig. 3. Inhibitory effects of HNF3β/FoxA2 expression levels via transfection of siRNA. (A) Assay for transfection efficiency with siRNA into ES cells. Alexa-labeled siRNA was transfected into ES cells in a manner identical to HNF3β siRNA transfection. The transfection efficiency was calculated as a percentage of Alexa-positive cells in total cells. Bars = 25 μm. (B) Real-time PCR analysis of HNF3β/FoxA2 gene via transfection of siRNA in HIFC-treated cells. Real-time PCR analysis was performed to examine HNF3β/FoxA2 expression from RNA extracted from differentiated ES cells transfected with either HNF3β/FoxA2 siRNA or luciferase-siRNA. HNF3β/FoxA2 expression levels were normalized to GAPDH expression levels. The mean ± SD of results from triplicate transfections is shown. Results represent the mean ± SD (n = 3). *$P > .05$. mRNA, messenger RNA; siRNA, small interfering RNA.

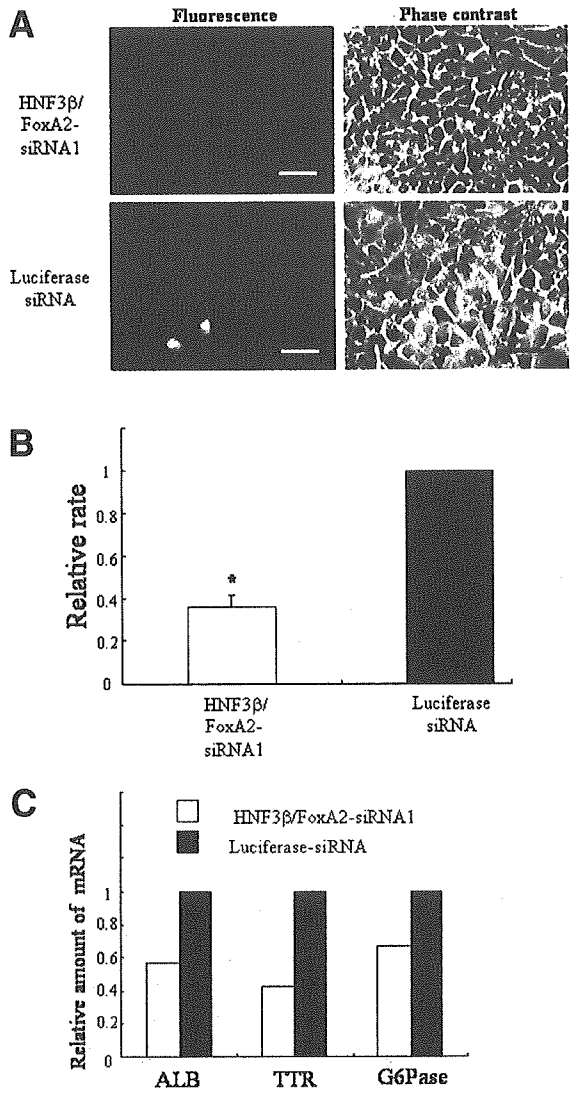

Fig. 4. Suppression of HNF3β/FoxA2 gene expression by siRNA affects the hepatic differentiation of ES cells. (A) Alteration of GFP-positive rates in differentiated ES cells transfected with either HNF3β/FoxA2 siRNA1 or luciferase siRNA. Bars = 10 μm. (B) HNF3β/FoxA2 siRNA1 was transfected with liposome on days 1 and 3 in the HIFC differentiation system. The GFP-positive rates were calculated as a percentage of GFP-positive cells in total cells on day 7. Results represent the mean ± SD (n = 3). *$P > .05$. (C) Effect of HNF3β/FoxA2 suppression on the mRNA levels of hepatocyte-specific genes including ALB, transthyretin, and glucose-6-phosphatase. The messenger RNA level of each gene was normalized to that of GAPDH, and the normalized messenger RNA level in the luciferase siRNA-transfected cells (control) was set to 1.0. siRNA, small interfering RNA; mRNA, messenger RNA; ALB, albumin; TTR, transthyretin; G6Pase, glucose-6-phosphatase.

gene networks require that ES cells differentiate into hepatocytes.

The expression of endoderm-associated transcription factor genes was detected in the early stage of the hepatic differentiation of ES cells. Previous reports showed that these transcription factors were also expressed in the visceral endoderm *in vivo* and induce transactivation of several endoderm- and hepatocyte-specific factors, including transthyretin, albumin, and L-pyruvate kinase.[24-28] Additionally, HNF4α is essential for morphological and functional differentiation of hepatocytes, accumulation of hepatic glycogen stores, and generation of the hepatic epithelium.[29] A previous report suggested that the cell maturation effect of oncostatin M is mediated through HNF4α in cultured human fetal hepatocytes.[30] RT-PCR analysis revealed that, in our system, HIFC-treated cells showed increased HNF4α expression critically at a late stage in the medium containing oncostatin M. Thus, our experimental data suggest that the HIFC differentiation system induces ES cells to differentiate into mature hepatocytes via the endoderm.

Visceral endoderm is an extraembryonic tissue derived from the inner cell mass of the embryo. It is functionally homologous to hepatocytes involved in lipid and glucose metabolism and serum protein production. Because of the difficulty in distinguishing visceral endoderm from embryonic endoderm, it is theoretically possible that our ES cell–derived hepatocytes might be hepatocyte-like cells from the visceral endoderm. However, a recent study demonstrated that the Cyp7A1 gene is expressed in hepatocytes derived from definitive endoderm, but not visceral endoderm.[31] In agreement with these findings, our HIFC-treated ES cell–derived hepatocytes expressed Cyp7A1 (data not shown). Additionally, based on both our previous[7] and present data, we have demonstrated via extensive characterization that our system produces the hepatocytes from mouse ES cells. Moreover, recent data from our laboratory has indicated that the HIFC system can be modified to allow differentiation of human mesenchymal stem cells into functional human hepatocytes (unpublished observation).

HNF3β/FoxA2 is known to be important in endoderm specification and subsequent hepatocyte differentiation *in vivo*.[26,32-34] On the other hand, no reports have shown its necessity in *in vitro* experiments because of the inability to differentiate ES cells into hepatocytes. In this study, HNF3β/FoxA2 siRNA transfection clearly repressed the differentiation into ALB-expressed cells. This result is in line with previous reports that HNF3β/FoxA2 is involved in early hepatocyte differentiation based on an *in vivo* animal model, suggesting that our HIFC differentiation system may recapitulate hepatic differentiation *in vivo* and may be useful for the study of a liver-specific transcriptional network.

Much is known about the molecular control of ectoderm and mesoderm development; however, little is known about endoderm regionalization and subsequent organ formation, which have been difficult to study *in vitro* because of the lack of a suitable model. According to

our data, the HIFC differentiation system allows ES cell differentiation into hepatocytes along a developmentally similar route. Previously, we reported that the HIFC differentiation system was critical for alpha-fetoprotein (AFP) gene expression at an early stage.[7] AFP is first detected in the gut endoderm at the four-somite stage of the mouse embryo.[35,36] In addition, AFP expression declines rapidly after birth, and the level of its messenger RNA in adult liver is less than 0.01% of that in fetal liver. During rapid hepatocyte proliferation, such as liver regeneration or tumorigenesis, AFP expression is reactivated. Hence, AFP might be a marker for liver progenitor cells or proliferating liver cells, such as oval cells.[37,38] Therefore, using this system, hepatic stem cells such as oval cells might be differentiated from ES cells and may be acquired abundantly. In support of this presumption, oval-like cell marker genes containing cytokeratin 19,[39] Thy-1,[40] γ-glutamyl transpeptidase,[41] and Dlk[42] are detectable using the HIFC differentiation system at early and middle stages (unpublished observations). Furthermore, this system is a valuable production model for the identification and characterization of factors involved in endoderm specification and hepatogenesis from hepatic stem cells. In the future, this system could be applied to the screening of key molecules for hepatic specification through an siRNA library approach.

In conclusion, the HIFC differentiation system closely mimics the actual *in vivo* hepatic developmental program: in development stage 1, pluripotent ES cells appear; in stage 2, endoderm markers, such as HNF3β/FoxA2, become positive; in stage 3, immature hepatocytes expressing ALB and AFP appear; and in stage 4, mature hepatocytes positive for ALB and tryptophan 2,3-dioxygenase are produced. This system could be a valuable tool for a variety of applications, such as toxicity testing, drug screening, study of the molecular biology of hepatitis B and C viruses, and production of therapeutic hepatocytes from human ES cells suitable for cell transplantation.

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References

1. Keller GM. In vitro differentiation of embryonic stem cells. Curr Opin Cell Biol 1995;7:862-869.
2. Rathjen J, Rathjen PD. Mouse ES cells: experimental exploitation of pluripotent differentiation potential. Curr Opin Genet Dev 2001;11:587-594.
3. Hromas R, Costa R. The hepatocyte nuclear factor-3/forkhead transcription regulatory family in development, inflammation, and neoplasia. Crit Rev Oncol Hematol 1995;20:129-140.
4. Pesce M, Scholer HR. Oct-4: gatekeeper in the beginnings of mammalian development. Stem Cells 2001;19:271-278.
5. Costa RH, Kalinichenko VV, Holterman AX, Wang X. Transcription factors in liver development, differentiation, and regeneration. HEPATOLOGY 2003;38:1331-1347.
6. Nagy P, Bisgaard HC, Thorgeirsson SS. Expression of hepatic transcription factors during liver development and oval cell differentiation. J Cell Biol 1994;126:223-233.
7. Teratani T, Yamamoto H, Aoyagi K, Sasaki H, Asari A, Quinn G, et al. Direct hepatic fate specification from mouse embryonic stem cells. HEPATOLOGY 2005;41:836-846.
8. Yamamoto H, Quinn G, Asari A, Yamanokuchi H, Teratani T, Terada M, et al. Differentiation of embryonic stem cells into hepatocytes: biological functions and therapeutic application. HEPATOLOGY 2003;37:983-993.
9. Quinn G, Ochiya T, Terada M, Yoshida T. Mouse flt-1 promoter directs endothelial-specific expression in the embyroid body model of embryogenesis. Biochem Biophys Res Commun 2000;276:1089-1099.
10. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, et al. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet 2001;29:365-371.
11. Haverty PM, Hansen U, Weng Z. Computational inference of transcriptional regulatory networks from expression profiling and transcription factor binding site identification. Nucleic Acids Res 2004;32:179-188.
12. Gonzalez FJ, Lee YH. Constitutive expression of hepatic cytochrome P450 genes. FASEB J 1996;10:1112-1117.
13. Kasik JW, Rice EJ. Transferrin gene expression in maternal liver, fetal liver and placenta during pregnancy in the mouse. Placenta 1993;14:365-371.
14. Shaw PH, Held WA, Hastie ND. The gene family for major urinary proteins: expression in several secretory tissues of the mouse. Cell 1983;32:755-761.
15. Zaret KS. Molecular genetics of early liver development. Annu Rev Physiol 1996;58:231-251.
16. Douarin NM. An experimental analysis of liver development. Med Biol 1975;53:427-455.
17. Fukuda-Taira S. Hepatic induction in the avian embryo: specificity of reactive endoderm and inductive mesoderm. J Embryol Exp Morphol 1981;63:111-125.
18. Houssaint E. Differentiation of the mouse hepatic primordium. I. An analysis of tissue interactions in hepatocyte differentiation. Cell Differ 1980;9:269-279.
19. Kamiya A, Kojima N, Kinoshita T, Sakai Y, Miyaijma A. Maturation of fetal hepatocytes in vitro by extracellular matrices and oncostatin M: induction of tryptophan oxygenase. HEPATOLOGY 2002;35:1351-1359.
20. Jones EA, Tosh D, Wilson DI, Lindsay S, Forrester LM. Hepatic differentiation of murine embryonic stem cells. Exp Cell Res 2002;272:15-22.
21. Ishizaka S, Shiroi A, Kanda S, Yoshikawa M, Tsujinoue H, Kuriyama S, et al. Development of hepatocytes from ES cells after transfection with the HNF-3beta gene. FASEB J 2002;16:1444-1446.
22. Hamazaki T, Iiboshi Y, Oka M, Papst PJ, Meacham AM, Zon LI, et al. Hepatic maturation in differentiating embryonic stem cells in vitro. FEBS Lett 2001;497:15-19.
23. Chinzei R, Tanaka Y, Shimizu-Saito K, Hara Y, Kakinuma S, Watanabe M, et al. Embryoid-body cells derived from a mouse embryonic stem cell line show differentiation into functional hepatocytes. HEPATOLOGY 2002;36:22-29.
24. Bossard P, Zaret KS. GATA transcription factors as potentiators of gut endoderm differentiation. Development 1998;125:4909-4917.
25. Levinson-Dushnik M, Benvenisty N. Involvement of hepatocyte nuclear factor 3 in endoderm differentiation of embryonic stem cells. Mol Cell Biol 1997;17:3817-3822.
26. Sund NJ, Ang SL, Sackett SD, Shen W, Daigle N, Magnuson MA, et al. Hepatocyte nuclear factor 3beta (Foxa2) is dispensable for maintaining the differentiated state of the adult hepatocyte. Mol Cell Biol 2000;20:5175-5183.
27. Duncan SA, Navas MA, Dufort D, Rossant J, Stoffel M. Regulation of a transcription factor network required for differentiation and metabolism. Science 1998;281:692-695.

28. Sasaki H, Hogan BL. Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. Development 1993;118:47-59.
29. Parviz F, Matullo C, Garrison WD, Savatski L, Adamson JW, Ning G, et al. Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. Nat Genet 2003;34:292-296.
30. Lazaro CA, Croager EJ, Mitchell C, Campbell JS, Yu C, Foraker J, et al. Establishment, characterization, and long-term maintenance of cultures of human fetal hepatocytes. HEPATOLOGY 2003;38:1095-1106.
31. Asahina K, Fujimori H, Shimizu-Saito K, Kumashiro Y, Okamura K, Tanaka Y, et al. Expression of the liver-specific gene Cyp7a1 reveals hepatic differentiation in embryoid bodies derived from mouse embryonic stem cells. Genes Cells 2004;9:1297-1308.
32. Guo Y, Costa R, Ramsey H, Starnes T, Vance G, Robertson K, et al. The embryonic stem cell transcription factors Oct-4 and FoxD3 interact to regulate endodermal-specific promoter expression. Proc Natl Acad Sci U S A 2002;99:3663-3667.
33. Ang SL, Rossant J. HNF-3 beta is essential for node and notochord formation in mouse development. Cell 1994;78:561-574.
34. Dufort D, Schwartz L, Harpal K, Rossant J. The transcription factor HNF3beta is required in visceral endoderm for normal primitive streak morphogenesis. Development 1998;125:3015-3025.
35. Gualdi R, Bossard P, Zheng M, Hamada Y, Coleman JR, Zaret KS. Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. Genes Dev 1996;10:1670-1682.
36. Tyner AL, Godbout R, Compton RS, Tilghman SM. The ontogeny of alpha-fetoprotein gene expression in the mouse gastrointestinal tract. J Cell Biol 1990;110:915-927.
37. Petersen BE. Hepatic "stem" cells: coming full circle. Blood Cells Mol Dis 2001;27:590-600.
38. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, et al. Bone marrow as a potential source of hepatic oval cells. Science 1999;284:1168-1170.
39. Shiojiri N, Lemire JM, Fausto N. Cell lineages and oval cell progenitors in rat liver development. Cancer Res 1991;51:2611-2620.
40. Petersen BE, Goff JP, Greenberger JS, Michalopoulos GK. Hepatic oval cells express the hematopoietic stem cell marker Thy-1 in the rat. HEPATOLOGY 1998;27:433-445.
41. Holic N, Suzuki T, Corlu A, Couchie D, Chobert MN, Guguen-Guillouzo C, et al. Differential expression of the rat gamma-glutamyl transpeptidase gene promoters along with differentiation of hepatoblasts into biliary or hepatocytic lineage. Am J Pathol 2000;157:537-548.
42. Tanimizu N, Nishikawa M, Saito H, Tsujimura T, Miyajima A. Isolation of hepatoblasts based on the expression of Dlk/Pref-1. J Cell Sci 2003;116:1775-1786.

Direct Hepatic Fate Specification From Mouse Embryonic Stem Cells

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The molecules responsible for hepatic differentiation from embryonic stem (ES) cells have yet to be elucidated. Here we have identified growth factors that allow direct hepatic fate-specification from ES cells by using simple adherent monolayer culture conditions. ES cell–derived hepatocytes showed liver-specific characteristics, including several metabolic activities, suggesting that ES cells can differentiate into functional hepatocytes without the requirement for embryoid body (EB) formation, *in vivo* transplantation, or a coculture system. Most importantly, transplantation of ES cell–derived hepatocytes in mice with cirrhosis showed significant therapeutic effects. In conclusion, this novel system for hepatic fate specification will help elucidate the precise molecular mechanisms of hepatic differentiation *in vitro* and could represent an attractive approach for developing stem cell therapies for treatment of hepatic disease in humans. *Supplementary material for this article can be found on the* HEPATOLOGY *website (http://www.interscience.wiley.com/jpages/0270-9139/suppmat.index.html).* (HEPATOLOGY 2005;41:836-846.)

Mouse embryonic stem (ES) cells are capable of differentiating into any adult animal cell type.[1,2] Although hepatocyte differentiation from mouse ES cells has been achieved[3-11] the utility of ES cells as (1) a developmental model and (2) a source for pharmaceutical screening and transplantation is hampered by limited control over the differentiation process. To address this limitation, we have established a highly reproducible *in vivo* method to acquire abundant functional hepatocytes from ES cells through liver-regenerating animals.[3] These hepatocytes express several differentiation markers of mature hepatocytes and showed sufficient functions to rescue experimental liver injury when transplanted *in vivo*. Our next goal was to elucidate the molecules responsible for directing this hepatic differentiation from ES cells, and to apply this information to developing a reproducible hepatic differentiation system *in vitro*.

To this end, we applied the results obtained from DNA-chip analysis to induce the direct differentiation of functional hepatocytes from an adherent monolayer culture of ES cells. These cells display the characteristics of mature hepatocytes apropos of liver-specific gene expression and biochemical analysis *in vitro*. Importantly, transplantation of monolayer-differentiated ES cell–derived hepatocytes improved liver function and prolonged the survival of mice with cirrhosis. Although the use of human ES cells for therapeutic purposes must be rigidly controlled, the procedure described in this study for inducing transplantable hepatic cells from mouse ES cells is, theoretically, transferable to human ES cells, leading to

Abbreviations: ES, embryonic stem; LIF, leukemia inhibitory factor; GFP, green fluorescent protein; HIFC, hepatic induction factor cocktail; RA, retinoic acid; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; PBS, phosphate-buffered saline; OsM, oncostatin M; HCM, hepatocyte culture medium; DMN, dimethylnitrosoamine; TTR, transthyretin; RT-PCR, reverse transcription-polymerase chain reaction; IGF, insulinlike growth factor, TGF, transforming growth factor; HNF, hepatocyte nuclear factor; ALB, albumin; TDO2, tryptophan 2,3-dioxygenase; AFP, alpha-fetoprotein; CK, cytokeratin; EB, embryoid body; HE, hematoxylin-eosin.

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the production of a valuable source of hepatocytes to treat liver disease in humans.

Materials and Methods

Culture of ES Cells. pALB-EGFP/ES cells,[3,12] a J1 cell clone of 129X1/SvJ male origin, were cultured on STO feeder cells with leukemia inhibitory factor (LIF) (Funakoshi, Tokyo, Japan) (+) medium to maintain pluripotency according to Wurst and Joyner.[13] After the removal of STO feeder cells, ES cells alone were plated on gelatin-coated plates (Asahi Techno Glass, Chiba, Japan). In a typical experiment, 5 to 8 gelatin-coated plates (100 mm in diameter) were seeded with 1×10^6 ES cells/plate and were cultured for further experiments with LIF (−) culture medium. Hepatic differentiation was identified by green fluorescent protein (GFP) expression, which was monitored by fluorescence microscopy (Nikon, Tokyo, Japan).

Microarray Analysis. We followed MIAME (minimum information about a microarray experiment) guidelines for the presentation of our data. Gene expression analysis was performed between CCl_4-treated and untreated mouse liver using complementary DNA microarrays (Affymetrix, Tokyo, Japan) to identify genes responsible for hepatic commitment. The procedure was conducted according to recommended protocols aspreviously described.[14] Microarrays were scanned using the GeneArray scanner (Affymetrix) at 3-μm resolution, and the scanned image was quantitatively analyzed by using Microarray Suite 4.0 software (Affymetrix). For normalizing of data to compare mRNA expression levels among samples, we arbitrarily selected 1,000 as an average of AD sources corresponding to the signal intensities of all probe sets in each sample, as recommended by the manufacturer.

In the gene expression analysis of ES cell–derived GFP-positive cells, we extracted RNA from day 0 (undifferentiated ES cells), day 10− (hepatic induction factor cocktail [HIFC] untreated sample) and day 10+ (HIFC treated sample) cells and 129X1/SvJ mouse liver (8 weeks old). As a reference total RNA, we mixed equal amounts of day 0, day 10−, and day10+ RNA. Hybridization and washing were performed according to the AceGene Mouse Oligo Chip Subset A and B protocol. The fluorescence intensities were scanned using ScanArray (PerkinElmer Life Sciences) and analyzed using DNASIS Array (Hitachi Soft, Kanagawa, Japan).

In Vitro *Culture.* pALB-EGFP/ES cells were cultured on gelatin-coated dishes for 3 days without feeder cells and treated for 3 days in ES cell culture medium containing LIF (100 units/mL) and 10^{-8} mol/L *all-trans*-retinoic acid (RA) (Step 1). After 3 days, the cells were passaged and cultured with LIF (−) culture medium for 5 days in the presence of fibroblast growth factor (FGF)1, 100 ng/mL; FGF4, 20 ng/mL; hepatocyte growth factor (HGF), 50 ng/mL (VERITAS, Tokyo, Japan) at 10^6 cells per 100-mm gelatin-coated plate (Step 2). Henceforth this mixture shall be referred to as hepatic induction factor cocktail (HIFC). Cells were then washed 3 times with cold phosphate-buffered saline (PBS) (−) and incubated for 20 minutes at 37°C in PBS containing 0.05% collagenase (GIBCO-BRL, Tokyo, Japan) and 1,000 units/mL dispase (Godoshusei, Tokyo, Japan). The dissociated cells were washed twice with serum-free Dulbecco's minimum essential medium (DMEM) and then resuspended in LIF (−) culture medium containing oncostatin M (OsM) (10 ng/mL) at 10^6 cells per 100 mm type I collagen-coated plate (Asahi Techno Glass) (Step 3). Two days after plating, cells were fed with hepatocyte culture medium (HCM), modified William E medium containing transferrin (5 μg/mL), hydrocortisone-21-hemisuccinate (10^{-6} mol/L), bovine serum albumin (0.5 mg/mL), ascorbic acid (2 mmol/L), insulin (5 μg/mL), and Gentamicin (50 μg/mL) (Sanko Junyaku, Tokyo, Japan) (Step 4). GFP gene expression was monitored by fluorescence microscopy. The GFP-positive rate was calculated as percentage of GFP-expressing cells per total cells in HIFC-treated plates. The matrices laminin, vitronectin, and fibronectin were used at 10 μg/mL, 6 μg/mL, and 1 μg/mL, respectively (Asahi Techno Glass). Mouse primary hepatocytes were cultured as described previously.[15] In brief, hepatocytes were isolated from male 129X1/SvJ mice weighing 20 g by means of the collagenase S-1 (Nitta Gelatin, Tokyo, Japan) perfusion method. The dispersed cells were suspended in HCM at 1.0×10^7 cells/mL and plated onto collagen-coated plates (Asahi Techno Glass) and cultured for a week before use. Between 95% and 97% of the harvested cells were viable and positive for albumin gene expression.

Animal Treatment and Cell Transplantation. Ten-week-old female 129X1/SvJ mice and BALB/c nude mice (SLC, Tokyo, Japan) were used in all studies. 1.0% dimethylnitrosamine (DMN) (Wako) dissolved in saline was injected intraperitoneally 3 consecutive days per week for 4 weeks into female 129X1/SvJ mice, and ES cell–derived GFP-positive hepatocytes (5×10^6 cells/0.1 ml/mouse) or control PBS(−) was given intravenously at day 28 after the initiation of DMN treatment. ES cell–derived GFP-positive hepatocytes were isolated by fluorescence-activated cell sorting. Each experimental group contained 10 mice. The mice were sacrificed periodically and dissected, and their livers were harvested and examined for the presence of GFP-positive fractions. Histological analysis of liver tissues was con-

ducted by serial tissue section at 1 and 21 days after cell transplantation. Both plasma fibrinogen and albumin expression were measured.

The firefly luciferase-expressing pALB-EGFP/ES cell line carrying pEGFPLuc plasmid DNA (CLONTECH, Tokyo, Japan) was established and used for *in vivo* imaging analysis of the fate of transplanted ES cells into mice with liver injury. *In vivo* bioimaging was conducted in a cryogenically cooled IVIS system (Xenogen), using living image acquisition and analysis software (Xenogen, Alameda, CA). The amount of light generated was directly related to the amount of luciferase-producing cells. The IVIS system could distinguish as few as 300 luciferase-positive ES cells *in vivo*.[15]

Electron Microscopy. To detect the hepatic cells differentiated from ES cells, GFP-positive fractions were observed by ordinary electron microscopy, as described previously.[3]

Immunohistochemical and Reverse Transcription Polymerase Chain Reaction Analysis. Cultured cells were washed twice with PBS(−) and fixed in 4.0% formaldehyde at 4°C for 10 minutes. Rehydrated sections were treated with 3.0% H_2O_2 in methanol for 15 minutes followed by incubation with 5% fetal calf serum in PBS for 30 minutes at 4°C. The cells were then analyzed by immunohistochemistry with transthyretin (TTR) (1:100) and CK18 (1:100) antibodies overnight at 4°C. The rhodamine isothiocyanate–conjugated secondary antibody (1:5,000) was applied for 30 minutes followed by incubation with 5% fetal calf serum. All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An aliquot of total RNA isolated from undifferentiated ES cells and GFP-positive cells using ISOGEN solution (Nippon Gene, Tokyo, Japan) was treated with DNase I (amplification grade; TaKaRa, Kyoto, Japan) according to the manufacturer's guidelines. Reverse transcription-polymerase chain reactions (RT-PCR) were performed by using a One-Step RT-PCR kit (QIAGEN, Tokyo, Japan). Primers and PCR details are available in Table 1.

Real Time-PCR Analysis. RNA expression normalized to the housekeeping gene GAPDH was measured by real-time PCR using the iCycler System (BIO-RAD, Tokyo, Japan) and Real-Time Detection System Software (Version 2.1). Real time-PCR monitoring was performed by adding the double-stranded DNA dye, SYBR Green I.

Biochemical Analyses. One day after plating at 2×10^5 cells/60-mm dish, ES cell–derived GFP-positive hepatocytes or control ES cells and normal mouse hepatocytes were analyzed for glucose levels in the culture supernatant by the glucose oxidase method, as described previously.[16] To examine the cellular activity of ammonia detoxification, GFP-positive cells or control ES cells and normal mouse hepatocytes were cultured at 2×10^5 cells/60-mm dish in 1.0 mL DMEM containing 2.5 mmol/L NH_4Cl and further incubated for 24 hours. The culture media were tested for concentration of NH_4Cl at 0, 6, 12, and 24 hours by Ammonia-Test Wako (Wako Pure Chemicals, Tokyo, Japan). To assay urea synthesis ability, cells were cultured with Hank's balanced salt solution in the presence of 5 mmol/L NH_4Cl. The medium was harvested after incubation for 0, 2, 4, and 6 hours and assayed according to the manufacturer's guidelines.

Blood samples were obtained periodically and centrifuged for 20 minutes at 5,000 rpm, and the serum was collected. Serum samples were tested for albumin concentration using the Albumin II-HA Test Wako kit (Wako Pure Chemicals). Plasma fibrinogen concentration was measured using the Drihemato Fib kit (Wako Pure Chemicals).

Statistical Analysis. The results are given as mean ± SEM. The Student t test was performed for statistical evaluation, with $P < .05$ considered significant. Where more than 2 samples were compared, the one-way ANOVA statistical analysis was performed, and results are given as ±SEM.

Table 1. Primers and Conditions for RT-PCR

GenBank Accession No.	Primers	Annealing Temperature (°C)	Cycles
AJ011413 ALB	S: 5′-CAGGATTGCAGACAGATAGTC-3′ A: 5′-GCTACGGCACAGTGCTTG-3′	56	32
BC018390 TDO2	S: 5′-TGCGCAAGAACTTCAGAGTGA-3′ A: 5′-AGCAACAGCTCATTGTAGTCT-3′	58	42
BC025934 TAT	S: 5′-ACCTTCAATCCCATCCGA-3′ A: 5′-TCCCGACTGGATAGGTAG-3′	56	36
BC024702 TTR	S: 5′-CTCACCACAGATGAGAAG-3′ A: 5′-GGCTGAGTCTCTCAATTC-3′	56	36
U00445 G6P	S: 5′-CAGGACTGGTTCATCCTT-3′ A: 5′-GTTGCTGTAGTAGTCGGT-3′	56	36
XM285741 CK8	S: 5′-ATGATGTACACCTCTGGCCC-3′ A: 5′-TCATATAGCTCTCTCCCCCA-3′	58	36
AB031959 Lst-1	S: 5′-TTGATGGGGAACATGCTTCG-3′ A: 5′-ACTTGCCATAGTGGGTATGG-3′	58	34
AF134407 CPS1	S: 5′-ATGACGAGGATTTTGACAGC-3′ A: 5′-CTTCACAGAAAGGAGCCTGA-3′	60	34
BC028342 PEPCK	S: 5′-TCTGCCAAGGTCATCCAGG-3′ A: 5′-GTTTTGGGGATGGGCACTG-3′	60	38
U04197 HNF3β	S: 5′-GCGAGTTAAAGTATGCTGGG-3′ A: 5′-CACTGATAGATCTCGCTCAG-3′	64	32
NM008261 HNF4α	S: 5′-ATTCTCCAACAGCCTGAGC-3′ A: 5′-CGTCTGTGATGTTGGCAATC-3′	64	34
V00743 AFP	S: 5′-TCGTATTCCAACAGGAGG-3′ A: 5′-AGGCTTTTGCTTCACCAG-3′	58	32
AB093573 ERas	S: 5′-TCTAGCATCTTGGACCTGAG-3′ A: 5′-TTCTTGCTTGATTCGGCCAC-3′	58	32
Y00864 c-Kit	S: 5′-CCCAAGACGTAACAGCTTCTG-3′	58	26

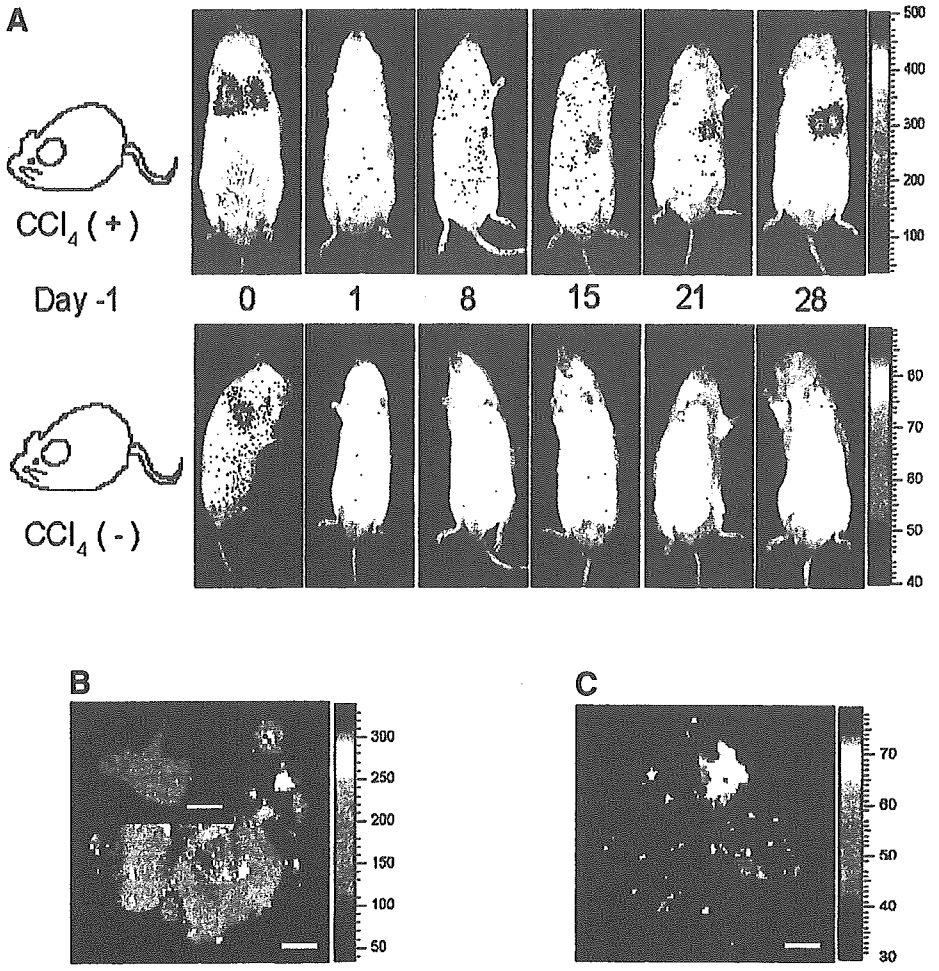

Fig. 1. Hepatic differentiation from ES cells. (A) *In vivo* imaging analysis of luciferase-expressing mouse ES cells transplanted into CCl_4-treated (+) (n = 3) or CCl_4-untreated (−) (n = 3) mice. All images shown are the visible light image superimposed on the optical CCD image with a scale in relative light units/min as shown. (B) Tumor formation was observed in the livers of CCl_4-administered mice at 3 weeks after the administration of CCl_4. The inset shows ES-derived tumors containing abundant GFP-positive cells (**scale bar:** 2.5 mm). Hepatocytes differentiated from pALB-EGFP/ES cells were recognized as GFP-positive cells.[3] No tumors were observed in the livers of the control mice without CCl_4-treatment. **Scale bars** represent 5.0 mm. ES, embryonic stem; GFP, green fluorescent protein.

Results

Identification of Hepatic Induction Factors. *In vivo* imaging analysis of luciferase-expressing mouse ES cells showed that transplanted ES cells homed specifically to the injured liver (Fig. 1A) and subsequently differentiated into GFP-positive cells (Fig. 1B, inset). As previously reported, tumor formation was observed in the liver of all CCl_4-treated mice.[3] Analysis of the tumors showed that 14% to 28% of the tissue comprised mature, transplanted hepatocytes. In contrast, transplanted mice with normal liver showed no such effects. These results indicate that the injured liver produces key regulatory factors that are necessary for the homing of ES cells to the site of liver injury and, additionally, for supporting hepatic differentiation. These observations led us to a rational design of differential-gene expression analysis between CCl_4-treated and untreated mouse liver. To this end, DNA microarray technology was used to identify genes responsible for hepatic differentiation. Initially, we focused specifically on induced growth factor genes, based on the pivotal role that growth factors appear to play in the differentiation of several cell types from ES cells (Table 2). Twelve species of growth factor genes, whose expression levels were undetectable in normal liver, were newly induced 24 hours after CCl_4-treatment; FGF-3, -4, -5, -8, -10, -13, -18, HGF, nerve growth factor-β (NGF-β), insulin-like growth factor (IGF)-2, transforming growth factor (TGF)α, and TGFβ2. In addition, the expression level of a further 5 species of growth factor genes were significantly increased in the regenerated liver (FGF-1: 1.8-, mast cell growth factor: 2.1-, hepatoma derived growth factor: 2.2-, IGF-1: 5.2-, and TGFβ1-4047μ: 1.8-fold). Furthermore, growth factor receptors for FGF (FGFR1, 3, and 4), IGF (IGF1R and 2R), oncostatin M receptor, and TGFβ (TGFβ1R and β2R) were also significantly upregulated.

Hepatic Differentiation of Mouse ES Cells in an Adherent Monolayer Culture. The 12 growth factors upregulated during liver regeneration were selected to investigate their effects on the hepatocyte differentiation from ES cells in adherent monoculture conditions. Undifferentiated pALB-EGFP/ES cells[3] whose GFP expres-

Table 2. GeneChip Analysis of Change-up in Growth Factor and Receptor Gene Expression in CCl_4-Treated Mouse Liver

Genbank Accession No.	Gene	Fold Change
M30641	FGF1	1.8
Y00848	FGF3	1.3*
X14849	FGF4	2.4*
M37823	FGF5	1.6*
D12483	FGF8	1.7*
D89080	FGF10	2.1*
AF020737	FGF13	1.7*
AB004639	FGF18	1.0*
X72307	HGF	1.8*
D63707	HDGF	2.2
M17298	βNGF	2.6*
X04480	IGF1	5.2
X71922	IGF2	1.3*
K01668	MCGF	2.1
M92420	TGFα	2.6*
AJ009862	TGFβ1	1.8
X57413	TGFβ2	1.6*
U22324	FGFR1	2.2*
M81342	FGFR3	1.4
X59927	FGFR4	1.1
AF056187	IGF1R	1.6*
U04710	IGF2R	1.6
AB015978	OsMR	3.1*
L15436	TGFβ1R	1.8*
D32372	TGFβ2R	1.1*

NOTE. Microarray analysis used human U95A oligonucleotide probe arrays (12,626 transcripts). The asterisk mark (*) indicates new expression og gene in CCl4-treated mouse liver. The numbers-fold increase for gene expression was measured by analyzer software (Affymetrix).

sion is driven by the mouse albumin promoter/enhancer were initially cultured on gelatin-coated plates without feeder cells and treated with ES cell culture medium containing LIF and RA for 3 days. At day 3, there were no GFP-positive cells detectable in the culture (data not shown). In the second stage of culturing, LIF and RA were removed from the medium, and cells were transferred to new gelatin-coated plates and exposed to various growth factors. Using the above culture system, 12 different recombinant growth factors were initially added individually to the culture medium (ESM)[3] during stage 2. Hepatic differentiation was directly identifiable as the GFP-positive cell fraction appearing, in some cases, as early as day 5 of culture (Fig. 2A). Among the 12 individual growth factors assayed, those that increased the numbers of GFP-positive cells were subjected to further analysis of synergistic effects in combination with other growth factors (Fig. 2B). We discovered that cells treated with a combination of FGF1, FGF4, and HGF, each of which is upregulated in the regenerating liver, consistently produced the most dramatic increase in GFP-positive cell numbers (22.9 ± 5.8%), which were scattered throughout the monolayer culture (Fig. 3C). Thus, the application of DNA-chip technology to our system provided an effective platform for identification of factors sufficient for establishment of direct hepatic fate specification from ES cells *in vitro*. OSM is thought to be effective for hepatic maturation,[17-19] and, interestingly, OSM receptor was upregulated in our DNA microarray analy-

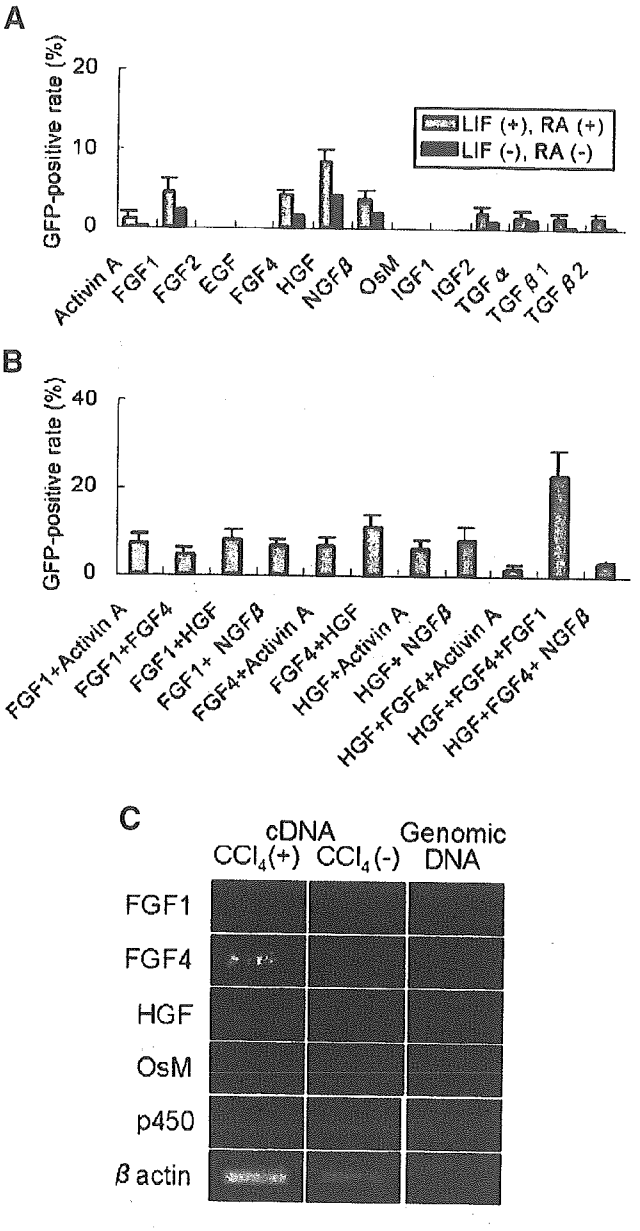

Fig. 2. Optimization of ES cells differentiation into hepatocytes. (A) Thirteen individual growth factors were tested for their ability to direct differentiation of hepatocytes from ES cells in adherent monoculture conditions (n = 5). (B) Among 11 different combinations, cells treated with FGF1, FGF4, and HGF produced the most significant numbers of GFP-positive cells (n = 5). (C) Expression of FGF1, FGF4, HGF, OsM, and p450 was analyzed by RT-PCR in the livers of CCl_4 (+) and (−) mice. Mouse genomic DNA from liver was used as a negative control. ES, embryonic stem; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; GFP, green fluorescent protein; NGF, nerve growth factor; EGF, epidermal growth factor; TGF, transforming growth factor ; OSM, oncostatin M; IGF, insulinlike growth factor.

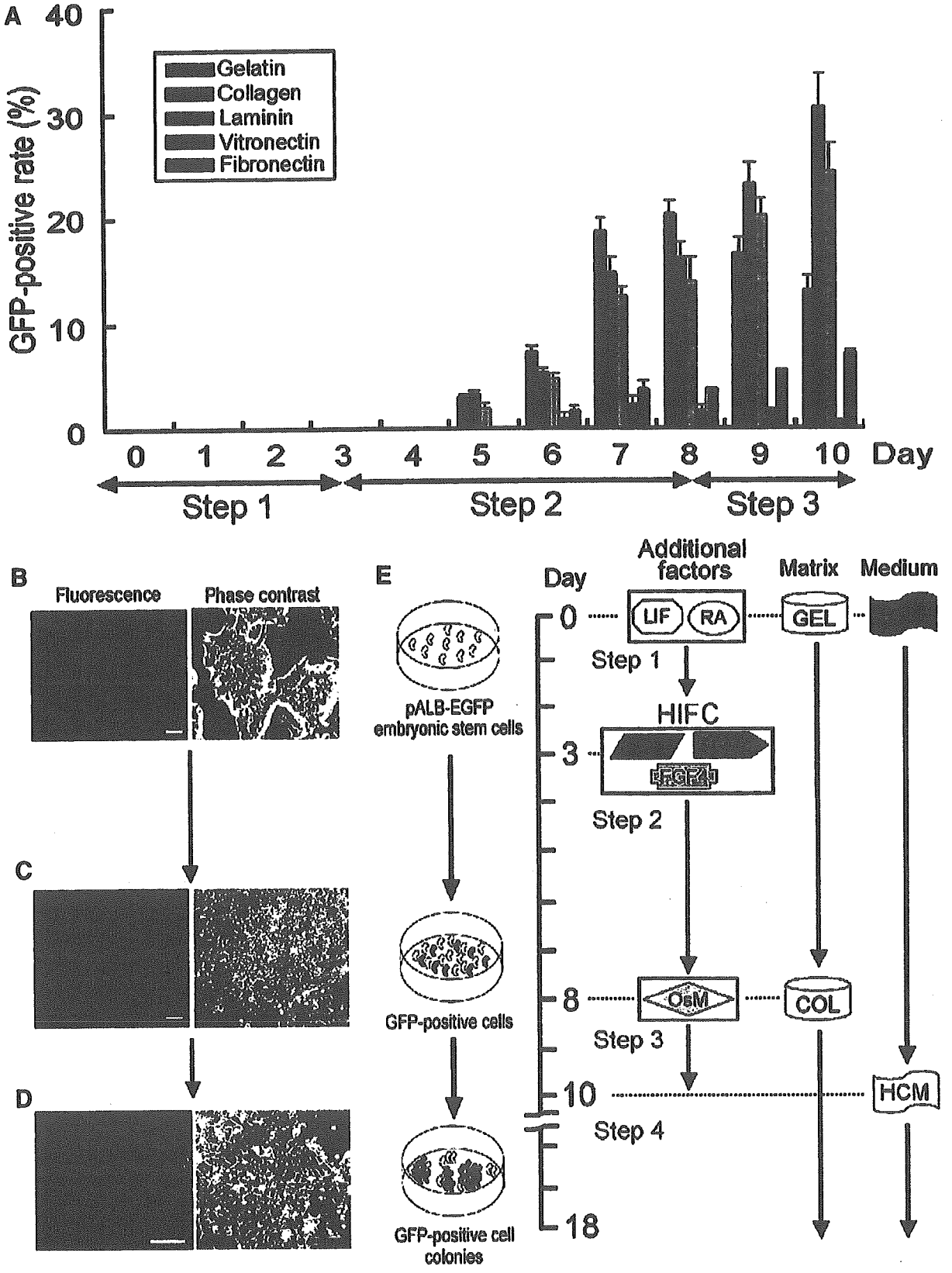

Fig. 3. Hepatic differentiation of mouse ES cells in adherent monoculture. (A) Kinetic analysis of GFP-positive cells cultured on a plate coated with several types of matrices. Data represent percentage of GFP-positive cells in total culture cells (N≥3). (B-D) Morphological changes and induction of GFP expression during the course of hepatocyte differentiation from ES cells: untreated (day 0) (B); the end of Step 2 (day 8) (C), 8 days after Step 4 (day 18) (D). **Scale bars** represent 50 μm. (E) An integrated schematic representation of the differentiation protocol for the induction of hepatocytes from ES cells in monolayer culture, steps 1 to 4 (see Materials and Methods) (n = 5). ES, embryonic stem; GFP, green fluorescent protein; LIF, leukemia inhibitory factor; RA, retinoic acid; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; OsM, oncostatin M; HCM, hepatocyte culture medium; ESM, ES medium.

sis. Five days after growth factor treatment and hepatic induction, OsM was assayed for its effect on hepatic differentiation by addition to the culture medium. OsM increased both GFP intensity and albumin production concomitantly in the GFP-positive cell fraction. However, the actual number of GFP-positive cells was not affected (data not shown).

Confirmatory analysis of gene expression profiles for FGF1, FGF4, HGF, and OsM in the regenerating liver were peformed by RT-PCR (Fig. 2C). Each of the assayed growth factor genes was detected and found to be upregulated in the regenerating liver as compared with the control, normal liver, thus verifying the data obtained by DNA-chip analysis. Taking these data together, we have elucidated a subset of growth factors sufficient for hepatic cell differentiation from ES cells *in vitro*, namely, FGF1, FGF4, and HGF.

Next, matrix-dependent hepatic cell growth was examined. After culturing with HIFC for 5 days on gelatin-coated dishes, the cells were trypsinized and replated on various matrices. Among 5 different extracellular matrix components assayed, type I collagen produced the highest yield of GFP-positive cells; 31.5 ± 6.3% at 7 days after culturing in the presence of OsM (Fig. 3A). GFP-positive cells production on the other matrices was as follows: gelatin 13.3 ± 2.0%; laminin at 24.4 ± 4.0%; fibronectin at 7.3 ± 0.3% and vitronectin at 0.7 ± 0.3%. These data show that the growth and enrichment sensitivity of ES cell–derived hepatocytes is sensitive to extracellular matrix component type and indicate that type I collagen may be an optimal substrate for hepatocyte induction

Thus, 2 days after OsM treatment, ES cell–GFP-positive cells were cultured with a serum-free HCM on type I collagen-coated plates for 3 weeks. Within 24 hours of replating, proliferating ES cell–derived GFP-positive cells were identified. Cells in these colonies proliferated 2 to 5 times in the first week and retained their GFP expression for a further 3 weeks in culture (Fig. 3D). Furthermore, the use of a serum-free HCM eliminated serum-dependent cells, nearly all of which were nonhepatic. After 2 weeks in culture, ES cell–derived GFP-positive cells stopped proliferating, even when split to a lower density, as might be expected for terminally differentiated hepatocytes. Thus, HCM culture conditions facilitate the enrichment of ES cell–derived GFP-positive cells up to 80% in culture. In summary, the following steps outline the strategy for maximal hepatic cell induction from ES cells: Step 1, RA and LIF treatment of ES cells for 3 days on gelatin-coated plates (endoderm induction stage); Step 2, HIFC treatment for 5 days on gelatin-coated plates (hepatic induction stage); Step 3, OsM treatment for 2 days on collagen-coated plates (hepatic maturation stage); Step 4, culture in serum-free HCM on collagen-coated plates (hepatocytes selection stage) (Fig. 3E). This differentiation program is highly efficient, with approximately 1×10^6 GFP-positive cells produced from 1×10^5 undifferentiated ES cells.

Characterization of GFP-Positive Cells. Microscopic analysis of ES cell–derived GFP-positive cells showed a hepatocyte-like morphology, with binucleate cells frequently observed (Fig. 4A-B). Electron microscopic analysis showed that large glycogen areas were often observed in epithelial cells of bile duct–like structures, which are abundant in the GFP-positive fractions (Fig. 4C). Additionally, ultrastructural characters of mitochondria (Mt) in the liver progenitor–like cells resemble those of mature hepatocytes. Immunogold particles indicating GFP molecules were detected in the cells containing bile canaliculi and peroxisomes (data not shown). We next ascertained whether ES cell–derived GFP-positive cells exhibit hepatocyte-specific gene expression profiles. ES cell–derived GFP-positive cells were collected by fluorescence-activated cell sorting, and RT-PCR analysis of sorted GFP-positive cells at Step 3 (see above) confirmed that the population expressed the liver-specific marker and enzyme genes: ALB, TDO2, TTR, TAT, G6P, CK8, Lst-1, CPS1, PEPCK, and p450 (CYP1A1), and transcriptional factor genes: hepatocyte nuclear factor (HNF)-3β (FoxA2) and -4α (see supplemental Fig. 1). This expression pattern is similar to that of primary hepatocytes. Importantly, GFP-positive cells were negative for c-*kit* and *ERas* gene expression which are both markers of undifferentiated ES cells and important for their tumor-like growth characteristics. The ES cell–derived GFP-positive cells also were negative for alkaline phosphatase activity, also a marker for undifferentiated ES cells (data not shown). In addition, CK19, an intrahepatic bile duct cell (cholangiocyte) marker, was not detected in ES cell–derived GFP-positive cells (data not shown), indicating that the ES cell–derived GFP-positive population does not contain other liver cell types. Kinetic and quantitative PCR analysis showed that albumin (ALB) gene expression was induced on the first day of Step 2 (see supplemental Fig. 1). Quantitative levels of ALB and tryptophan 2,3-dioxygenase (TDO2) mRNA expression were normalized to the internal reference gene GADPH at day 0, day 6, and day 10. ALB mRNA concentration increased gradually from day 6 to day 10, and TDO2 expression was detectable at day 10, but not at day 0 or day 6, by real-time PCR analysis. The level of ALB mRNA at day 6 was approximately 20% of levels in ES cell–derived GFP-positive cells at day 10. Before ALB expression, alpha-fetoprotein (AFP) gene expression was identified on the second day of Step 1, reached maximal expression levels at the end of Step 1, and diminished thereafter. TDO2, a specific marker of mature hepatocytes, was identified in

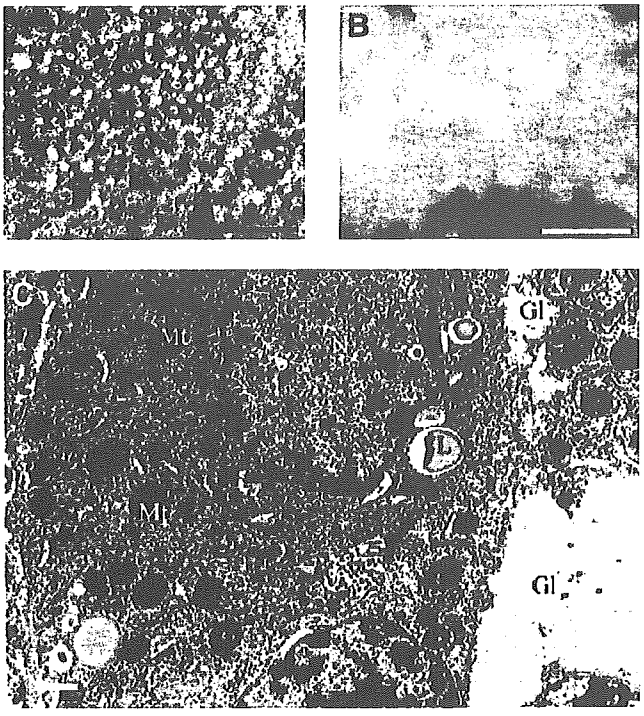

Fig. 4. Morphological characteristic of GFP-positive cells. (A) **Arrow** indicates binucleate cell in phase contrast. (B) Fluorescence image. **Scale bars** represent 50 μm. (C) Electron microscopy of the GFP-positive fractions. Gl, glycogen areas; Mt, mitochondria; bc, bile canaliculi; N, nuclear; L, lipid granule. **Scale bars**, 0.5μm. GFP, green fluorescent protein.

the late stage of Step 2, reached maximal expression levels at Step 3, and subsequently retained its expression in HCM for several weeks. Immunohistochemical staining for hepatocyte-specific proteins indicated that HNF3β was positive at day 3. GFP-positive cells were double positive for cytokeratin (CK)18/ALB and TTR/ALB at day 10 (Fig. 5). We next compared liver-specific gene expression profiles between ES cell–derived hepatocytes and normal mouse liver by DNA-chip analysis, using microarrays from Mouse Genome Informatics (http://www.informatics.jax.org/). Differentiated, ES cell–derived hepatocytes and normal mouse liver revealed a 98% correspondence of gene expression (number of genes assayed = 88) (see supplemental Table 1). Biochemical analyses indicated that cultured GFP-positive cells display glucose-producing ability, capacity to clear ammonia from the culture media, and urea synthesis ability, characteristics of mature hepatocytes (see supplemental Fig. 2). Moreover, levels of glucose and urea produced by ES cell–derived GFP-positive cells were similar to those produced by monolayer cultures of primary hepatocytes. Conversely, HIFC-untreated ES cells did not produce glucose and urea and lacked the capacity to clear ammonia from the culture media. Together, these biochemical data indicate that our ES cell–derived GFP-positive cells are functional hepatocytes. Karyotyping of more than 40 ES cell–derived GFP-positive cells by G-banding analysis showed that all cells had the normal chromosome number (data not shown). In addition, no teratoma formation was observed when 1×10^6 GFP-positive cells cultured for 1 week were subcutaneously injected into BALB/c nude mice (n = 6) and 129X1/SvJ mice (n = 4). Thus, hepatocytes differentiated from ES cells by our culture system display the phenotypic and biochemical characteristics and functional activity of normal mature hepatocytes.

Transplantation of GFP-Positive Cells Into Mice With Cirrhosis. Finally, to address our ultimate goal of examining whether these hepatocytes are therapeutically applicable, we transplanted 5×10^6 GFP-positive cells per mouse into mice with DMN-induced cirrhosis. The transplanted ES cell–derived GFP-positive cells were immediately diffused through the liver and integrated adjacent to the fibrotic region, along with the expression of GFP (Fig. 6A-B, arrows). By counting the number of GFP-positive cells in 100 tissue sections, the number of GFP-positive cells that migrated into the host liver was estimated to be at least 1×10^6 cells per mouse. Histo-

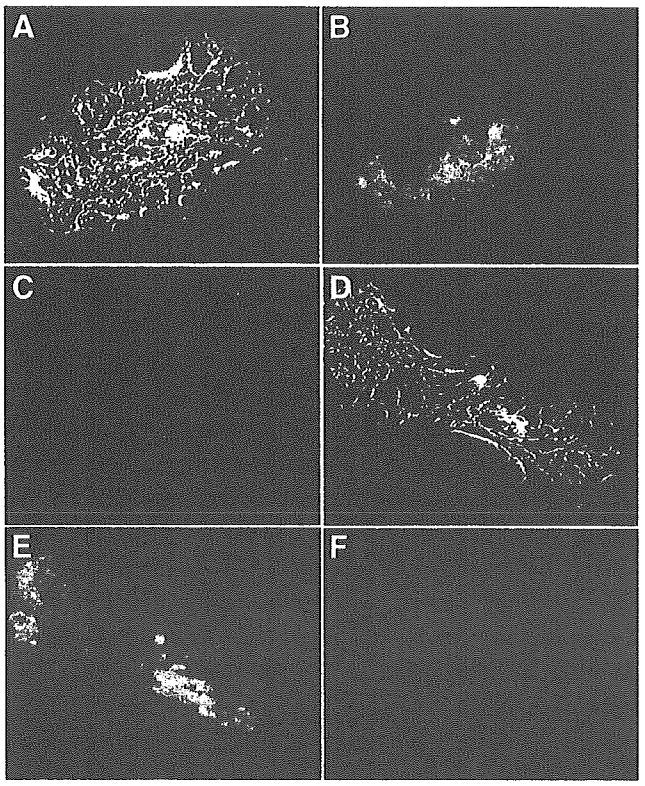

Fig. 5. Immunohistochemical analysis of GFP-positive cells. (A, D) Phase contrast of ES cell-derived-hepatocytes. (B, E) Fluorescent images of isolated ES cell-derived GFP-positive hepatocytes in hepatocyte culture medium and analyzed at 2 weeks after step 3. (C, F) Immunofluorescent images of CK18 (C) and TTR (F) staining in ES cell-derived ALB-positive hepatocytes. ES, embryonic stem; GFP, green fluorescent protein; TTR, transthyretin.

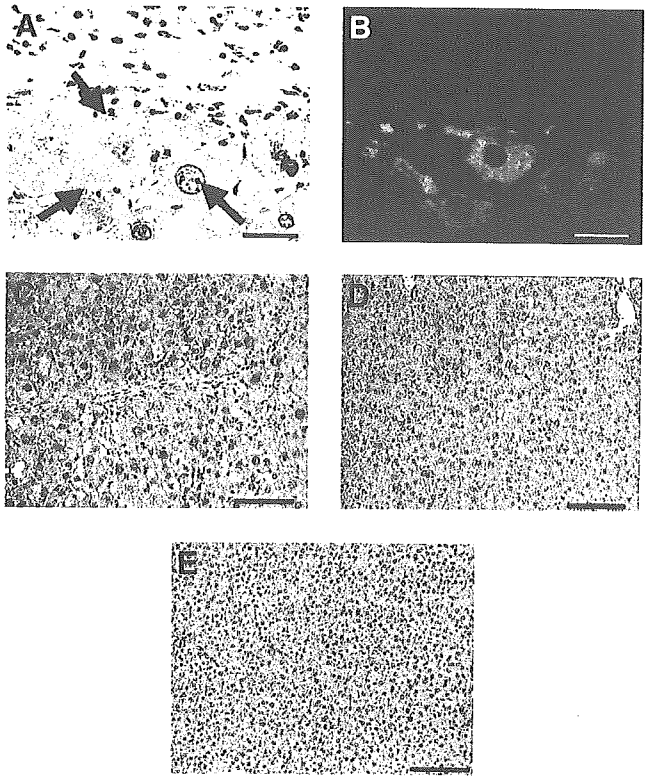

Fig. 6. Transplantation of ES cell-derived GFP-positive cells into mice with DMN-induced cirrhosis. (A) Hematoxylin-eosin (HE) staining of liver sections 1 day after the transplantation of ES cell-derived GFP-positive hepatocytes. (B) Grafted cells, indicated by **arrows** in (A), were detected by GFP fluorescence in the same section. (C) HE staining of liver section from control DMN-treated mice administered with saline (21 days after treatment). (D) HE staining of liver section from DMN-treated mice administered with ES-derived hepatocytes (21 days after transplantation). (E) HE staining of liver sections from age-matched, untreated normal mice. **Scale bars** represent 10 μm (A, B) and 50 μm (C-E). ES, embryonic stem; GFP, green fluorescent protein; DMN, dimethylnitrosoamine.

logical analysis indicated that transplantation of ES cell–derived GFP-positive cells significantly suppressed the onset of fibrosis/cirrhosis in mice (Fig. 6C-E). Survival analysis showed that mice receiving GFP-positive cells survived for at least twice as long as control mice injected with PBS(−) alone (Fig. 7A). In addition, plasma fibrinogen and albumin concentrations were increased 1.6- and 1.3-fold, respectively, over those of the control mice administered with PBS(−) (Fig. 7B-C). No teratoma or liver tumor formation was observed at 80 weeks after the transplantation of GFP-positive cells into either normal 129X1/SvJ or liver-damaged mice (n = 10) (Fig. 8). Thus, our ES cell–derived hepatocytes functioned therapeutically *in vivo*, and their transplantation ameliorated the effects of DMN-induced cirrhosis.

Discussion

Recent reports have highlighted the differentiation of hepatocytes from ES cells *in vitro*[5,6,8-11] and *in vivo*.[3,4,7] To elucidate the molecular mechanisms underlying the development of the liver, previous reports have attempted the differentiation of ES cells into hepatocytes by forming embryoid bodies (EBs) *in vitro*. However, because at least some of the cells of EBs are not terminally differentiated, this material is not useful for transplantation. Moreover, EB differentiation is not a scalable process. Nevertheless, none of these articles have clarified the precise molecular mechanisms of hepatic induction, especially in regards to what kind of growth signals are required for hepatocyte differentiation from ES cells. To address this question, we de-

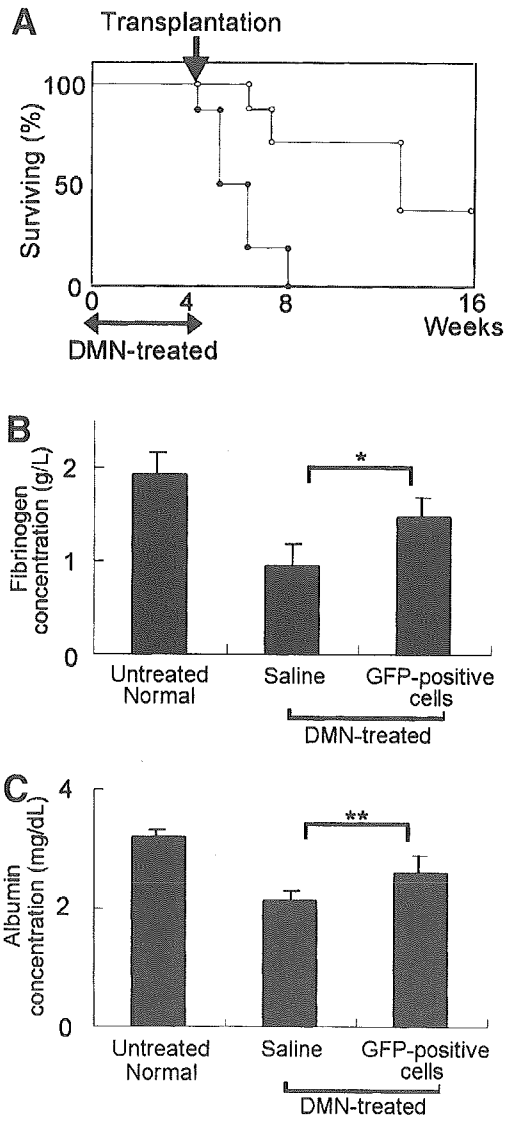

Fig. 7. *In vivo* functions of ES cell-derived hepatocytes. (A) Survival curve of mice with DMN-induced cirrhosis. The **arrow** indicates time of transplantation of ES cell-derived GFP-positive hepatocytes (**open circles**: mice administered with ES cell-derived GFP-positive hepatocytes; **closed circles**: control mice administered with saline). Plasma fibrinogen (B) and albumin (C) concentrations were measured in the same mice. N = 10 in each experimental group. ES, embryonic stem; GFP, green fluorescent protein; DMN, dimethylnitrosoamine.

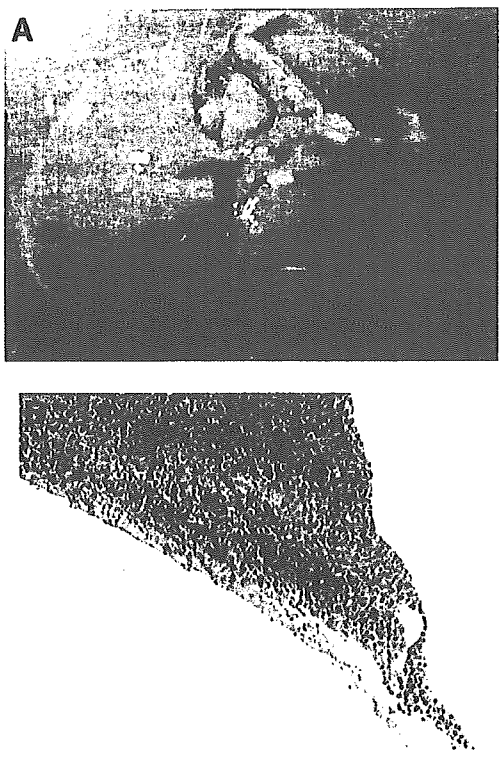

Fig. 8. Transplantation of ES cell-derived GFP-positive cells into mice with cirrhosis at 80 weeks. (A) No tumor was observed in the transplantation of ES cell-derived hepatocytes into mice with DMN-induced cirrhosis (n = 10). (B) Hematoxylin-eosin (HE) staining of a serial section from A. ES, embryonic stem; GFP, green fluorescent protein; DMN, dimethylnitrosoamine.

duced the growth factors that direct hepatic fate specification and applied them to establish a methodology for the direct differentiation of functional hepatocytes from an adherent monoculture of ES cells. These cells display the characteristics of mature hepatocytes with respects to liver-specific gene expression and functionality *in vitro*. More importantly, transplantation of our ES cell–derived hepatocytes improved liver function and prolonged survival in a clinically relevant model of cirrhosis. We had previously shown that hepatic cells were efficiently induced when ES cells were transplanted into mice after liver injury.[3] Although these hepatocytes are chromosomally normal, for clinical application of ES cell–derived hepatocytes, *in vitro* differentiation of hepatocytes from ES cells is imperative from a quality control standpoint. In the current study, we have advanced to successfully induce the direct differentiation of functional mature hepatocytes without the use of animals or the formation of EB, which are, hence, more suitable for clinical use. Although the application of human ES cells for therapeutic purposes is currently subject to regional regulatory restrictions, the induction of transplantable hepatic cells from mouse ES cells described here may be instantly transferable to human ES cells, leading to the production of human hepatocytes. In support of this, differentiation of hepatocytes from *Cynomolgus monkey* ES cells is achievable using HIFC (T. Teratani, unpublished observation).

The immortality and rapid growth of ES cells are attractive features for their use in stem cell therapies.[20-22] However, the finding that ES cells produce teratomas when transplanted in syngenic animals[23] is likely to preclude their therapeutic usage.[24-26] In our system, although serum-free HCM allowed enrichment of hepatocytes to almost 80% in the culture dish, the remaining 20% fraction contains non-hepatic cells, including other types of differentiated cells and, potentially, immature ES cells. However, our ES cell–derived hepatocytes neither formed skin tumors when subcutaneously injected into athymic nude mice and 129X1/SvJ mice nor displayed anchorage independence in soft agar. Additionally, ERas message, the transforming oncogene important in the teratoma-forming ability of ES cells,[27] was undetectable in the ES cell–derived hepatocytes. Nevertheless, although cell sorting may increase the purity of hepatocytes up to 98%, there is a finite risk that residual undifferentiated ES cells could form teratomas when they are implanted into a recipient. Such a risk, when balanced against the therapeutic benefits of stem cell therapy, might be managed by the use of human *ERas*-knockout human ES cells.

It was recently reported that bone marrow–derived adult stem cells differentiated into hepatocyte-like cells *in vitro*.[28] However, bone marrow–derived adult stem cells may also repair damaged liver by cell fusion within the host liver and not by converting directly into hepatic cells.[29-31] Although possibly induced fusion of cells may be therapeutically exploited to achieve the rescue of damaged liver tissue, problems are likely to arise because the fusion mechanisms are not fully understood. In this regard, it may be preferable to use ES cells as a source of functional hepatocytes rather than adult stem cells for treating human diseases. Finally, during mouse liver development, AFP and ALB are differentially regulated. AFP synthesis starts soon after fertilization, whereas the earliest time ALB can be detected is at day 17 or 18.[32,33] In our system, HIFC-stimulated AFP mRNA expression preceded ALB mRNA, indicating that ES cell–derived hepatocytes mimic the normal liver developmental program. Additionally, during embryonic liver development, late stage, hematopoietic cells produce OsM, which induces maturation of mouse fetal hepatocytes[17,18] and induces the differentiation of cultured human fetal hepatocytes.[19] In our system, OsM was critical for G6P and TDO2 expressions at late stage and an increase of GFP intensity. Thus, our system was shown the supporting of these papers. Furthermore, HNF3β (FoxA2) expression is important for the endoderm lineage,[34] and HNF4α is essential for morphological and functional dif-

ferentiation of hepatocytes, accumulation of hepatic glycogen stores, and generation of the hepatic epithelium.[35] These important transcription factor genes, for HNF4α and HNF3β (FoxA2), were expressed in our system through to the final differentiation stage. Thus, our *in vitro* hepatic cell induction system appears to mimic *in vivo* hepatic development and will therefore be useful for studying regulatory mechanisms of hepatocyte-specific and liver-enriched transcription factor gene expression.

In conclusion, this study documents an experimentally deduced combination of growth factors and matrixes that is sufficient for reproducible and efficient hepatic differentiation leading to direct induction of mature functional hepatocytes from ES cells in adherent monoculture conditions, with therapeutic efficacy. Our system will be a valuable tool for studying the molecular basis of the developmental processes influencing hepatic cells *in vitro* and bring us a step closer to establishing a safe and effective stem cell therapy to treat hepatic failure *in vivo*.

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References

1. Keller GM. In vitro differentiation of embryonic stem cells. Cur Opin Cell Biol 1995;7:862-869.
2. Rathjen J, Rathjen PD. Mouse ES cells: experimental exploitation of pluripotent differentiation potential. Curr Opin Genet Dev 2001;11:587-594.
3. Yamamoto H, Quinn G, Asari A, Yamanokuchi H, Teratani T, Terada M, et al. Differentiation of embryonic stem cells into hepatocytes: biological functions and therapeutic application. HEPATOLOGY 2003;37:983-993.
4. Choi D, Oh HJ, Chang UJ, Koo SK, Jiang JX, Hwang SY, et al. In vivo differentiation of mouse embryonic stem cells into hepatocytes. Cell Transplant 2002;11:359-368.
5. Jones EA, Tosh D, Wilson DI, Lindsay S, Forrester LM. Hepatic differentiation of murine embryonic stem cells. Exp Cell Res 2002;272:15-22.
6. Chinzei R, Tanaka Y, Shimizu-Saito K, Hara Y, Kakinuma S, Watanabe M, et al. Embryonic-body cells derived from a mouse embryonic stem cell line show differentiation into functional hepatocytes. HEPATOLOGY 2002;36:22-29.
7. Yin Y, Lim YK, Salto-Tellez M, Ng SC, Lin CS, Lim SK. AFP(+), ESC-derived cells engraft and differentiate into hepatocytes in vivo. Stem Cells 2002;20:338-346.
8. Ishizaka S, Shiroi A, Kanda S, Yoshikawa M, Tsujinoue H, Kuriyama S, et al. Development of hepatocytes from ES cells after transfection with the HNF-3beta gene. FASEB J 2002;16:1444-1446.
9. Miyashita H, Suzuki A, Fukao K, Nakauchi H, Taniguchi H. Evidence for hepatocyte differentiation from embryonic stem cells in vitro. Cell Transplant 2002;11:429-434.
10. Hamazaki T, Iiboshi Y, Oka M, Papst PJ, Meacham AM, Zon LI, et al. Hepatic maturation in differentiating embryonic stem cells in vitro. FEBS Lett 2001;497:15-19.
11. Yamada T, Yoshikawa M, Kanda S, Kato Y, Nakajima Y, Ishizaka S, et al. In vitro differentiation of embryonic stem cells into hepatocyte-like cells identified by cellular uptake of indocyanine green. Stem Cells 2002;20:146-154.
12. Quinn G, Ochiya T, Terada M, Yoshida T. Mouse flt–1 promoter directs endothelial-specific expression in the embyroid body model of embryogenesis. Biochem Biophys Res Commun 2000;276:1089-1099.
13. Wurst W, Joyner AL. Production of targeted embryonic stem cell clones. In: Joyner AL, ed. Gene Targeting. Washington, DC: IRL Press, 1990:33-61.
14. Aoyagi K, Tatsuta T, Nishigaki M, Akimoto S, Tanabe C, Omoto Y, et al. A faithful method for PCR–mediated global mRNA amplification and its integration into microarray analysis on laser-captured cells. Biochem Biophys Res Commun 2003;300:915-920.
15. Vooijs M, Jonkers J, Lyons S, Berns A. Noninvasive imaging of spontaneous retinoblastoma pathway-dependent tumors in mice. Cancer Res 2002; 62:1862-1867.
16. Sistare FD, Haynes RC Jr. The interaction between the cytosolic pyridine nucleotide redox potential and gluconeogenesis from lactate/pyruvate in isolated rat hepatocytes: implications for investigations of hormone action. J Biol Chem 1985;260:12748-12753.
17. Kamiya A, Kojima N, Kinoshita T, Sakai Y, Miyajima A. Maturation of fetal hepatocytes in vitro by extracellular matrices and oncostatin M: induction of tryptophan oxygenase. HEPATOLOGY 2002;35:1351-1359.
18. Kamiya A, Kinoshita T, Miyajima A. Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways. FEBS Lett 2001;492:90-94.
19. Lazaro CA, Croager EJ, Mitchell C, Campbell JS, Yu C, Foraker J, et al. Establishment, characterization, and long-term maintenance of cultures of human fetal hepatocytes. HEPATOLOGY 2003;38:1095-1106.
20. Kaufman DS, Thomson JA. Human ES cells-haematopoiesis and transplantation strategies. J Anat 2002;200:243-248.
21. Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 2001;19:1129-1133.
22. Bjorklund LM, Sanchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, et al. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. Proc Natl Acad Sci U S A 2002;99:2344-2349.
23. Hilberg F, Wagner EF. Embryonic stem (ES) cells lacking functional c-jun: consequences for growth and differentiation, AP-1 activity and tumorigenicity. Oncogene 1992;7:2371-2380.
24. Yanai J, Doetchman T, Laufer N, Maslaton J, Mor-Yosef S, Safran A, et al. Embryonic cultures but not embryos transplanted to the mouse's brain grow rapidly without immunosuppression. Int J Neurosci 1995;81:21-26.
25. Freed CR. Will embryonic stem cells be a useful source of dopamine neurons for transplant into patients with Parkinson's disease? Proc Natl Acad Sci U S A 2002;99:1755-1757.
26. Wakitani S, Takaoka K, Hattori T, Miyazawa N, Iwanaga T, Takeda S, et al. Embryonic stem cells injected into the mouse knee joint form teratomas and subsequently destroy the joint. Rheumatology 2003;42:162-165.
27. Takahashi K, Mitsui K, Yamanaka S. Role of ERas in promoting tumour-like properties in mouse embryonic stem cells. Nature 2003;423:541-545.
28. Schwartz RE, Reyes M, Koodie L, Jiang Y, Blackstad M, Lund T, et al. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest 2002;109:1291-1302.
29. Medvinsky A, Smith A. Stem cells: fusion brings down barriers. Nature 2003;422:823-825.
30. Vassilopoulos G, Wang PR, Russell DW. Transplanted bone marrow regenerates liver by cell fusion. Nature 2003;422:901-904.
31. Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, et al. Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 2003;422: 897-901.
32. Camper SA, Tilghman SM. The activation and silencing of gene transcription in the liver. Biotechnology 1991;16:81-87.
33. Nahon JL. The regulation of albumin and alpha-fetoprotein gene expression in mammals. Biochimie 1987;69:445-459.
34. Lai E, Prezioso VR, Tao W, Chen WS, Dranell JE Jr. Hepatocytes nuclear factor 3 alpha belongs to a gene family in mammals that is homologous to the Drosophila homeotic gene fork hand. Genes Dev 1993;5:416-427.
35. Parviz F, Matyllo C, Garrison WD, Savatski L, Adamson JW, Ning G, et al. Hepatocytes nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. Nat Genet 2003;34:292-296.